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(57) Abstract

A new class of synthetic glycosulfopeptides (GSPs) which have one or more sulfated tyrosine residues and a glycan linked to the peptide, the glycan preferably including a sialyl Lewis^x group or a sialyl Lewis^a group. In a preferred version the GSPs have an O-glycan comprising a β1,6 linkage to a GalNAc. The present invention further contemplates *in vitro* methods of the synthesis of these GSPs without the use of the cells and methods of their use *in vivo* as powerful anti-inflammatory antithrombotic, or anti-metastatic compounds. The invention also contemplates a method of synthesizing oligosaccharides by cleaving the glycan from the GSP.

GLYCOSULFOPEPTIDES AND METHODS OF SYNTHESIS AND USE THEREOF

BACKGROUND

The present invention is directed to glycosulfopeptides, methods of their synthesis, and methods of their use in treating inflammation.

Inflammation is the reaction of vascularized tissue to local injury. This injury can have a variety of causes, including infections and direct physical injury. The inflammatory response can be considered beneficial, since without it, infections would go unchecked, wounds would never heal, and tissues and organs could be permanently damaged and death may ensue. However, the inflammatory response is also potentially harmful. Inflammation can generate pathology associated with rheumatoid arthritis, myocardial infarction, ischemic reperfusion injury, hypersensitivity reactions, and some types of fatal renal disease. The widespread problem of inflammatory diseases has fostered the development of

many "anti-inflammatory" drugs. The ideal drug would be one that enhances the good effects resulting from the inflammatory response, and at the same time prevents the potentially harmful side-effects of this response.

The inflammatory response in regard to blood cells is accompanied by adhesion of circulating neutrophils, the most abundant phagocytic cell in the blood, to activated endothelial cells that line the vessels and make up the vessel walls. The adherent neutrophils are subsequently activated and the activated neutrophils emigrate from the blood into the surrounding tissue in a process termed diapedesis. The cells then begin engulfing microorganisms in a process termed phagocytosis and they also degranulate, releasing a variety of degradative enzymes, including proteolytic and oxidative enzymes into the surrounding extracellular environment. The mechanisms by which neutrophils adhere, become activated, and emigrate from the blood are currently major topics of research around the world. It is hoped that a fundamental understanding of these mechanisms will give rise to a new generation of anti- and pro-inflammatory drugs and treatments.

The initial attraction of circulating leukocytes to sites of inflammation is due to binding of the cells to a class of adhesion molecules termed selectins. The three currently identified selectins are L-selectin, which is constitutively expressed on the surfaces of all circulating leukocytes; E-selectin which is inducibly expressed on the surfaces of endothelial cells; and P-selectin, which is inducibly expressed on the surfaces of platelets

and endothelial cells. The selectins recognize counter-receptors on other cells and thereby mediate cell-to-cell adhesive contacts. For example, P-selectin binds to a constitutively expressed, mucin-like glycoprotein counter-receptor on neutrophils termed the P-selectin glycoprotein ligand-1 (PSGL-1). the interaction between P-selectin and PSGL-1 promotes tethering and rolling adhesion of neutrophils on the vessel wall leading to neutrophil activation and eventual tight adhesion and diapedesis via integrins and their counter-receptors. Since it is well established that the selectin-mediated adhesion is an essential prelude to neutrophil activation and emigration during the inflammatory response, a tremendous amount of research has been done to identify compounds that inhibit neutrophil adhesion.

There have been attempts to use sialyl Lewis^x mimetics to control or regulate the inflammatory response via inhibition of selectin-mediated adhesion [Lowe, "Therapeutic Inhibition of Carbohydrate-protein Interactions *In Vivo*," J. Clin. Invest., 100(11 Suppl):S47-51, 1997]. These are modified simple carbohydrates (<2,000 daltons) that contain the sialyl Lewis^x or sialyl Lewis^a antigen [Varki, "Sialic Acids As Ligands In Recognition Phenomena," FASEB Journal, 11(4):248-55, 1997]. The sialyl Lewis^x mimetics have been made as either free carbohydrates or as adduct between the carbohydrates and lipids to alter their solubility properties. Synthesis of these mimetics has been by one of two routes. In one common method the carbohydrate mimetics have been produced by entirely chemical steps beginning with commonly

available precursors and organic chemical approaches. In the other common method the carbohydrate portions of the sialyl Lewis^x mimetics have been synthesized primarily using recombinant or partly purified glycosyltransferases, including sialyltransferases, galactosyltransferases, fucosyltransferases and sugar nucleotide donors, such as cytosinemonophosphate sialic acid, uridinediphospho galactose and guanosinediphospho fucose. In all cases, the efficacy of these sialyl Lewis^x mimetics has been poor and high doses (>0.5 mM) of the compounds are required, because they do not accurately reflect the structure of the appropriate selectin counter-receptor, e.g., PSGL-1, for P-selectin.

PSGL-1 on human leukocytes contains at its extreme amino terminus tyrosine residues that can potentially be sulfated and threonine residues that are potential binding sites for attachment of O-glycans containing N-acetylgalactosamine, N-acetylglucosamine, galactose, fucose and sialic acid with the sequence of the sialyl Lewis x antigen (McEver et al., "Leukocyte Trafficking Mediated by Selectin-Carbohydrate Interaction", J. of Biol. Chem., 270:11025-8, 1995. McEver et al., "Role of PSGL-1 Binding to Selectins in Leukocyte Recruitment", J. of Clin. Invest., 100:485-491, 1997).

The co-expression of sulfated tyrosine residues and the O-glycan appears to be required for high affinity interactions between PSGL-1 and P-selectin. However, naturally-occurring quantities of PSGL-1 are limited and it is not feasible to produce PSGL-1 from human neutrophils in a form suitable for

administration as an anti-inflammatory compound. Moreover, recombinant means of synthesis of PSGL-1 are tedious and expensive and require animal cell culture and the consequent problems and uncertainties of proper post-translational modifications of the PSGL-1 peptide backbone, of which tyrosine sulfate and O-glycan addition are examples. It would be desirable to have a process which enables the formulation and production of compounds which overcome these problems.

DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B are a schematic which describes a method of synthesis of a glycosulfopeptide in accordance with the present invention.

In Figures 1A and 1B, glycosulfopeptide AcGP-1 is represented by SEQ ID NO:6, GP-1 by SEQ ID NO:7, GP-2 by SEQ ID NO:8, GP-3 by SEQ ID NO:9, GP-4 by SEQ ID NO:10, GP-5 by SEQ ID NO:11, GP-6 by SEQ ID NO:12, and GSP-6 by SEQ ID NO:13.

Figures 2A and 2B are a schematic which describes an alternate method of synthesis of a glycosulfopeptide in accordance with the present invention.

In Figures 2A and 2B, glycosulfopeptide P-1 is represented by SEQ ID NO:14, GP-1 by Seq ID NO:7, GP-2 by SEQ ID NO:8, GP-3 by SEQ ID NO:9, GP-4 by SEQ ID NO:10, GP-5 by SEQ ID NO:11, GP-6 by SEQ ID NO:12, and GSP-6 by SEQ ID NO:13.

Figures 3A and 3B are a schematic which describes yet another alternate method of synthesis of a glycosulfopeptide in accordance with the present invention.

In Figures 3A and 3B, glycosulfopeptide AcGSP-1 is represented by SEQ ID NO:15, GSP-1 by SEQ ID NO:16, GSP-2 by SEQ ID NO:17, GSP-3 by SEQ ID NO:18, GSP-4 by SEQ ID NO:19, GSP-5 by SEQ ID NO:20, and GSP-6 by SEQ ID NO:13.

Figure 4 is a schematic which describes yet another alternate method of synthesis of a glycosulfopeptide in accordance with the present invention.

In Figure 4, glycosulfopeptide AcGSP-2-1 is represented by SEQ ID NO:21, GSP-2-1 is represented by SEQ ID NO:22, GSP-2-2 is represented by SEQ ID NO:23, GSP-2-3 is represented by SEQ ID NO:24, and GSP-2-4 is represented by SEQ ID NO:25.

Figure 5 is a graph showing the effects of various compounds on adhesion of neutrophils to immobilized soluble P-selectin.

Figure 6A, 6B and 6C show chemical structures of a number of R groups among those which may comprise the O-glycan portion of the glycosulfopeptides contemplated by the present invention.

Figure 7 shows formulas of glycosulfopeptides contemplated by the present invention wherein the R groups represented are those in Figure 6.

Figure 8 shows formulas of alternative embodiments of glycosulfopeptides contemplated by the present invention wherein the R groups are those represented in Figures 6A-6C.

Figure 9 shows formulas of additional alternative embodiments of glycosulfopeptides contemplated by the present invention wherein the R groups represented are those in Figures 6A-6C.

Figures 10A and 10B show specific amino acid sequences for a number of glycosulfopeptides contemplated herein, where the glycosulfopeptides may comprise from one to three sulfates and R groups R_1-R_{15} as defined in Figures 6A-6C.

In Figures 10A and 10B glycosulfopeptide A is represented by SEQ ID NO:26, B by SEQ ID NO:27, C by SEQ ID NO:28, D by SEQ ID NO:29, E by SEQ ID NO:30, F by SEQ ID NO:31, G by SEQ ID NO:32, H by SEQ ID NO:33, I by SEQ ID NO:34, J by SEQ ID NO:35, K by SEQ ID NO:36, L by SEQ ID NO:37, M by SEQ ID NO:38 and N by SEQ ID NO:39.

Figure 11 shows a formula of a glycosulfopeptide comprising a core-1 O-glycan. Core-1-Glycosulfopeptide-6 is represented by SEQ ID NO:40.

DESCRIPTION OF THE INVENTION

The present invention contemplates a new class of synthetic glycosulfopeptides (GSPs) which mimic the extreme amino terminus of PSGL-1 in that they comprise one or more sulfated tyrosine residues and a glycan comprising a sialyl Lewis^x group or a sialyl Lewis^a group. In a preferred embodiment the GSPs further comprise an O-glycan comprising a ß1,6 linkage to a GalNAc. The present invention further contemplates *in vitro* methods of the synthesis of these GSPs without the use of cells and methods of their use *in vivo* as powerful anti-inflammatory antithrombotic, or anti-

metastatic compounds which are able to block the selectin-mediated adhesion of cells [Kim et al., "P-Selectin Deficiency Attenuates Tumor Growth and Metastasis," Proc. Natl. Acad. Sci. USA, 95(16):9325-30, 1998].

The glycosulfopeptides generated herein could not have been derived easily by expression of recombinant glycoproteins. Precise definition of the glycosylation and tyrosine sulfation in recombinant glycoproteins is exceedingly difficult, because of the inherent heterogeneity in glycan structures and the variable efficiency of tyrosine sulfation. Furthermore, cellular initiation of O-glycosylation by α-GalNAcT is complex. Each of the many different enzymes in this family may require a slightly different peptide motif for initiation of O-glycosylation in cells. The novel technology contemplated herein for synthesis of glycosulfopeptides allows the complete control of the O-glycan sites and structures without regard to O-glycosylation motifs. The *in vitro* synthesis of these glycosulfopeptides as contemplated herein also allows the introduction of modified monosaccharides, e.g., sialic acid derivatives, precise modifications of glycan structures, and modifications in the peptide length.

While the invention will now be described in connection with certain preferred embodiments in the following examples so that aspects thereof may be more fully understood and appreciated, it is not intended to limit the invention to these particular embodiments. On the contrary, it is intended to cover all alternatives, modifications and equivalents as may be included

within the scope of the invention as defined by the appended claims. Thus, the following examples, which include preferred embodiments will serve to illustrate the practice of this invention, it being understood that the particulars shown are by way of example and for purposes of illustrative discussion of preferred embodiments of the present invention only and are presented in the cause of providing what is believed to be the most useful and readily understood description of formulation procedures as well as of the principles and conceptual aspects of the invention.

Examples

Example 1

In one embodiment, represented in Figures 1A and 1B, the present invention contemplates glycosulfopeptides and a method of synthesis thereof by enzymatically adding sugars and sulfate groups to a presynthesized peptide comprising at least one tyrosine residue and at least one natural or synthetic amino acid residue able to provide an O-glycosidic linkage (e.g., serine, threonine, hydroxyproline, tyrosine, hydroxylysine, methionine). The peptide preferably comprises from two amino acids to 30 amino acids, and more particularly may comprise from 3 to 29 amino acid residues, 4 to 28 amino acid residues, 5 to 27 amino acid residues, 6 to 26 amino acid residues, 7 to 25 amino acid residues, 8 to 24 amino acid residues, 9 to 23 amino acid residues, 10 to 22 amino acid residues, 11 to 21 amino acid residues, 12 to 20 amino acid

residues, 13 to 19 amino acid residues, 14 to 18 amino acid residues, 15 to 17 amino acid residues, or 18 amino acid residues. In the exemplary peptide shown in Figures 1-4, 10, and 11, the amino acid sequence is the same as amino acids 42-63 of PSGL-1 except for an additional glycine residue added to the N-terminal end of residue 42.

The glycosulfopeptide preferably comprises at least one sulfated tyrosine residue, more preferably two sulfated tyrosine residues, and most preferably three sulfated tyrosine residues. Each tyrosine residue is preferably separated by at least one additional amino acid residue.

In this embodiment, the peptide is synthesized on a commercial peptide synthesizer using the f-moc or t-boc derivative of tetraacetylated GalNAc in α linkage to the hydroxyl group of the O-linking residue. This α-linked derivative of the O-linked amino acid is inserted at the desired position of the peptide during synthesis of the peptide thereby adding a tetraacetylated GalNAc with the O-linking residue.

The tetraacetylated GalNAc is then "unblocked" in step (1) by deacetylating with a weak base in organic solvent, such as sodium methoxide. The specific introduction of a GalNAc residue at the desired position ensures that the sugar-linked amino acid is present in quantitative and stoichiometric levels and that no other O-linked residue in another position in the peptide is modified.

In the next step (2), Gal is β-linked to the GalNAc via β1,3-GalT in the presence of UDPGal. In the next step (3), GlcNAc is β-

linked to the GalNAc via β1,6-GlcNAcT in the presence of UDPGlcNAc. In the next step (4), Gal is β-linked to the GlcNAc via β1,4-GalT in the presence of UDPGal. In the next step (5), NeuAc is α-linked to the Gal via α2,3-ST in the presence of CMPNeuAc. In the next step (6), fucose is α-linked to the GlcNAc via α1,3-FT in the presence of GDPfucose. In the last step (7) of the synthesis, a sulfate group is added to each of the one or more tyrosine residues in the peptide via recombinant or at least partially purified TPST in presence of PAPsulfate. These steps are described in more detail in the experimental procedures section below. The result of the synthetic process in this embodiment is a peptide comprising a sulfated tyrosine and a sialyl Lewis^x group having a β1,6 linkage to a GalNAc residue in O-linkage to a serine or threonine (or a similarly O-linkable amino acid). As noted above, in this embodiment, it is not possible to preferentially link a GalNAc to a specific threonine (or serine) residue (if there is more than one in the peptide). Further, although N-acetyl neuraminic acid is the preferred sialic acid to be used, other sialic acids which function in a similar manner are contemplated to be used in the glycosulfopeptides claimed herein. These alternative sialic acids include other sialic acids which can be transferred via the enzyme α2,3-ST, including N-glycolylneuraminic acid, N-acetylneuraminic acid, 9-O-acetyl-N-glycolylneuraminic acid, 9-O-acetyl-N-acetylneuraminic acid and other sialic acids described in Varki et al., "Sialic Acids As Ligands In Recognition Phenomena", <u>FASEB</u>

Journal, 11(4):248-55, 1997, which is hereby incorporated by reference herein.

In this embodiment, it is also not possible to specifically sulfate only a particular tyrosine residue if the peptide comprises more than one tyrosine residue. Embodiments wherein it is possible to specifically sulfate particular tyrosine residues are described below.

A key to the abbreviations used herein is provided below.

DETAILED EXPERIMENTAL PROCEDURES:

Synthesis of the Glycosulfopeptide of Example 1

Crude glycopeptide 1 (GP-1) was synthesized at the Protein Resource Facility of Oklahoma State University. Tri-O-acetylated GalNAc was incorporated into the peptide during the solid phase synthesis using tri-O-acetyl-GalNAcαFmoc Thr derivative (Oxford GlycoSciences, Oxford, UK). The crude GP-1 (2 mg) was de-O-acetylated with 6 mM methanolic sodium methylate as described in Medal et al., Int. J. Peptide Protein Res., 43:529-536, 1994. The deacetylated peptide was purified by reversed phase HPLC. The retention time of deacetylated GP-1 (34.6 min) was clearly different from the tri-O-acetylated GP-1 (45.3 min). The yield of the pure GP-1 was 1.1-1.5 mg. In matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectra, the observed m/z for the $[M-H]^-$ molecular ion was 2952.9 (calc m/z 2953.2). In addition, the $[M-H]^-$ molecular ion of oxidized GP-1 (m/z 2969.5) was present where the methionine residue had been oxidized.

GP-1 was galactosylated overnight at 37°C in 100-200 nmol aliquots by using 3-4 times molar excess of UDPGal (Sigma) and 13 nmol/h of purified core-1 β1,3-GalT (core-1 β1,3-galactosyl transferase) in a total volume of 100 μl of 50 mM MES, pH 6.5, 2 mM ATP, 15 mM $MnCl_2$, 0.2% Triton X-100. The complete amino acid sequence of core-1 β1,3-GalT is shown in SEQ ID NO:1. After removing proteins and Triton X-100 by chloroform-methanol (2:1) extraction (deproteination), the reaction mixture was analyzed by HPLC. The retention time of the galactosylated product, GP-2, was 32.9 min and the degree of galactosylation was >95%. MALDI-TOF analysis of GP-2 revealed *m/z* 3115.4 for the $[M-H]^-$ molecular ion (calc. *m/z* 3115.3).

GP-2 (0.4-0.6 mM) was incubated at 37°C with 1-2 mM UDPGlcNAc (Sigma) and affinity purified recombinant core-2 β1,6-GlcNAcT (100 nmol/h) in a total volume of 100 μl of 50 mM Na-cacodylate, pH 7.0. A method for obtaining core-2β1,6-GlcNAcT is described below. After 24 h incubation a small aliquot from the reaction mixture was analyzed by HPLC. GP-2 was converted quantitatively into a faster moving product, GP-3 (retention time 31.3 min). MALDI-TOF mass spectrum of GP-3 showed *m/z* 3318.2 for the $[M-H]^-$ molecular ion (calc. *m/z* 3318.5). The reaction mixture was taken directly to a β1,4-GalT reaction. Alternatively, UDP[^{3}H]GlcNAc (American Radiolabeled Chemicals Inc., St. Louis, MO) (12,000 cpm/nmol) was used as a donor in the core-2 β1,6-GlcNAcT reaction to get [^{3}H]GP-3.

GP-3 (0.4 mM) (core-2 β1,6GlcNAcT reaction mixture) was galactosylated using 125 mU of bovine milk β1,4-GalT (Sigma) and UDPGal (1.5 mM) in a total volume of 160 μl of 40 mM Na-cacodylate, pH 7.0, 20 mM $MnCl_2$ and 0.02% NaN_3. After 20 h incubation at 37°C a sample from the reaction mixture was analyzed by HPLC, which showed that all GP-3 had been converted into a faster moving product, GP-4 (retention time 30.4 min). In MALDI-TOF analysis the observed *m/z* for the $[M-H]^-$ molecular ion of GP-4 was 3480.4 (calc. *m/z* 3480.7). Glycopeptide samples were deproteinated and desalted in a Sephadex G-50 column (10 ml, 0.7x25 cm) using water or 25 mM NH_4HCO_3 as an eluant. 0.5 ml fractions were collected and the glycopeptides were detected by measuring either UV absorbance at 215 nm or radioactivity of the fractions. After desalting and deproteination the sample was taken directly to an α2,3-sialylT reaction. Radiolabeled $[^3H]$GP-3 was galactosylated using UDP$[^3H]$Gal (Amersham, Buckinhamshire, England) (10,000 cpm/nmol) as a donor.

GP-4 (1 mM) was sialylated using 20 mU of α2,3-(N)-sialylT (Calbiochem, La Jolla, CA) and 3 mM CMPNeuAc (Sigma) in a total volume of 50 μl of 50 mM MOPS, pH 7.4, 0.1% BSA and 0.02% NaN_3. After 14 h incubation at 37°C a 1 μg sample was analyzed by HPLC, which showed that GP-4 had been converted completely into a faster moving product, GP-5 (retention time 29.7 min). In MALDI-TOF analysis the observed *m/z* for the $[M-H]^-$ molecular ion of GP-5 was 3770.6 (calc. *m/z* 3771.9). The reaction mixture was used directly for the α1,3-FucT reaction. Radiolabeled $[^3H]$GP-4 (0.1 mM) was

also sialylated using the donor CMP[^{3}H]NeuAc (0.2 mM, 31,500 cpm/nmol) (NEN, Boston, MA).

GP-5 (0.4 mM) was α1,3-fucosylated for 16 h at 37°C with 2 mU of α1,3-FucT-VI (Calbiochem, La Jolla, CA) and GDPFuc (0.8 mM) (Calbiochem, La Jolla, CA) in a total volume of 120 μl of 50 mM MOPS, pH 7.4, 20 mM $MnCl_2$ and 0.02% NaN_3. A deproteinated and desalted sample was analyzed by HPLC which showed that GP-5 was converted completely into the product GP-6 (retention time 29.1 min). In MALDI-TOF analysis the observed m/z for the $[M-H]^-$ molecular ion of GP-6 was 3917.5 (calc. m/z 3918.1). Starting with 185 μg of GP-2 the overall recovery of GP-6 was 88 μg, as determined by UV absorbance at 275 nm during the HPLC runs. Radiolabeled [^{3}H]GP-4 was fucosylated using GDP[^{14}C]Fuc (83,000 cpm/nmol) (Amersham) as the donor.

Several aliquots of GP-6 (0.02 mM) were sulfated for 35 h at 37°C using 0.15 mM PAPS (Sigma) or [^{35}S]PAPS (NEN, Boston, MA) (specific activity 30300 cpm/nmol) and 0.85 nmol/h of recombinant human TPST-1 (SEQ ID NO:2). Alternatively, hTPST-2, (SEQ ID NO:3) could be used or any other functional tyrosylprotein sulfotransferase. The total reaction volume was 100 μl per aliquot in 40 mM PIPES, pH 7.0, 0.05 M NaCl, 0.1% Triton X-100 and 5 mM EDTA. After chloroform-methanol (2:1) extraction to remove protein and detergent, the reaction mixture was desalted by gel filtration and subjected to HPLC. The retention time of the product, GSP-6, was 15.6 min and the conversion of GP-6 to GSP-6 was >95%. Electrospray mass spectrum analysis showed the molecular mass of

GSP-6 as 4158.0 (calc. 4159.2), confirming that three sulfate groups were present.

Alternatively, a radiolabeled form of GSP-6 was generated by incubating GP-6 (0.01 mM) for 14-17 h with 0.06 mM [^{35}S]PAPS (107,000-559,000 cpm/nmol) and 0.36 nmol/h of TPST-1 in a total volume of 50 μl. The conversion of GP-6 to $^{35}SO_3$-GSP-6 was >85%. GP-2 (0.08 mM) was sulfated for 35 h at 37°C by using 0.6 mM PAPS (Sigma) and 4.8 nmol/h of affinity purified recombinant TPST-1 in a total reaction volume of 400 μl. After deproteination and desalting the sample was analyzed by HPLC. The retention time of the product was 21.4 min and the conversion of GP-2 to GSP-2 was 98%. Electrospray mass spectra of GSP-2 showed the molecular mass as 3356.0 (calc. 3356.5), which confirmed that three sulfate groups were present. Alternatively, GP-2 (0.04 mM) was sulfated for 18 h at 37°C using [^{35}S]PAPS (0.2 mM, 30300 cpm/nmol) (Sigma) and TPST-1 (0.7 nmol/h) in a total volume of 56 μl. The conversion of GP-2 to $^{35}SO_3$-GSP-2 was >95%. GP-5 was sulfated in a similar fashion as GP-6 using [^{35}S]PAPS (30300 cpm/nmol) as a donor. The conversion of GP-5 to $^{35}SO_3$-GSP-5 was >90%. The retention time of $^{35}SO_3$-GSP-5 was 17.5 min in HPLC (not shown).

Reversed Phase High Performance Liquid Chromatography

Glycopeptide samples were filtered on a Spin-X membrane (Corning Costar, Cambridge, MA) and were subsequently analyzed in a reversed phase C-18 HPLC column (Vydac, Hesperia, CA) on a Beckman System Gold HPLC. The following solvent system was used at

P-selectin Affinity Chromatography

Soluble P-selectin was coupled to Ultralink® Biosupport Medium (Pierce, Rockford, IL) according to manufacturer's instructions. P-selectin columns (0.8 ml, 0.6 x 2.7 cm) of different densities (0, 1.0, 1.3, 1.6, 2.0 mg/ml) were equilibrated with 25 ml of buffer A. Radiolabeled glyco(sulfo)peptides (800-1000 cpm, 1-10 cpm, 1-10 pmol) were dissolved in 200 μl of buffer A and applied to the sPS-columns. Bound material was eluted with Buffer B (20 mM MOPS, pH 7.5, containing 10 mM EDTA, 150 mM NaCl, 0.02% NaN_3). Fraction size was 0.5 ml and the flow rate was 200-250 μl/min. All fractions were counted for radioactivity.

Mass Spectrometric Analysis

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was performed in the linear negative ion delayed extraction mode with a Biflex® time-of-flight instrument (Bruker-Franzen Analytik, Germany) equipped with a nitrogen laser operating at 337 nm. HPLC purified glycopeptide samples, except GSP-2 and GSP-6, were dissolved in 30% aqueous acetonitrile, and a 0.5 μl aliquot (about 2.5 pmol) was mixed with 0.5 μl of 2,4,6-trihydroxyacetophenone matrix (3 mg/ml in acetonitrile/20 mM aqueous diammonium citrate, 1:1, by vol.) and immediately dried under reduced pressure. The spectra were externally calibrated with insulin $[M-H]^-$ and $[M-2H]^{2-}$ signals. Electrospray ionization (ESI) mass spectra were collected in the negative ion mode using a Q-TOF hybrid quadrupole/orthogonal

acceleration time-of-flight mass spectrometer (Micromass Ltd., UK). GSP-2 and GSP-6 were dissolved in 50% aqueous acetonitrile and injected into the mass spectrometer with a nanoelectrospray ion source. Instrument calibration was performed with sodium trifluoroacetate ion clusters.

Desialylation of GSP-6

$^{35}SO_3$-GSP-6 (6,000 cpm) was desialylated by treatment with 8.4 mU of *Arthrobacter ureafaciens* neuraminidase (Sigma) in 100 μl of 0.1 M sodium acetate pH 5.5 for 13 h at 37°C. The reaction mixture was desalted and deproteinated before analysis by chromatography on sPS-columns.

Construction, Expression and Purification of Recombinant, Soluble Core-2 β1,6-GlcNAcT

A fusion protein was constructed that contained the catalytic and stem region of human core 2 β1,6-GlcNAcT with the 12-amino acid HPC4 epitope at both the N- and C-termini. The epitope is bound in a Ca^{2+}-dependent manner by the monoclonal antibody HPC4 [Rezaie et al., Prot. Exp. Purif., 3:453-460, 1992]. The catalytic and stem region of the core 2 β1,6-GlcNAcT was amplified by PCR using a pcDNA3 plasmid containing the full length cDNA of the human core 2 β1,6-GlcNAcT (type L) as a template (Bierhizen et al., Proc. Natl. Acad. Sci. USA, 89:9326-9330, 1992). The following primers were used for amplification:

5' primer containing BamHI cleavage site, 5'-GCCTGAATTTGTAAGGGATCCACACTTAGAGCTTGCTGGGGAGAATCC-3' (SEQ ID NO:4) and 3'-primer containing EcoRI site and HPC4 epitope, 5'-GTAGAATTCTTATCACTTGCCGTCGATCAGCCTGGGGTCCACCTGGTCCTCGTGTTTTAATGTCTCCAAAGC-3' (SEQ ID NO:5). The PCR product (1.2 kb) was cloned into pCR-TOPO 2.1 vector (Invitrogen, Carlsbad, CA) and used to transform *E. coli* strain JM109 for plasmid preparation. The construct was released from pCR-TOPO 2.1 vector by digestion with BamHI and Eco RV and purified by agarose gel electrophoresis. The construct (1.2 kb) was ligated into a BamHI/Eco RV site of modified pcDNA 3.1(+) vector (pcDNA 3.1 (+)TH) which contains an NH_2-terminal transferrin signal sequence and HPC4 epitope (Ouyang et al., Proc. Natl. Acad. Sci USA, 95:2896-2901, 1998) and used to transform *E. coli* strain DH5α. The resulting plasmid, pcDNA 3.1(+)TH-sC2 (6.7 kb), was isolated, sequenced and used to transfect CHO/dhfr$^-$ cells using lipofectamine (Life Technologies, Inc.) Clonal selection was carried out under neomycin resistance, and the cells were maintained in low salt DME (Cellgro, Herndon, Virginia) containing 10% fetal calf serum and G418 (600 μg/ml). Stable clones of cells expressing core 2 β1,6-GlcNAcT activity in the media (50 nmol/h/ml) were selected and grown to 100% confluency. The media was changed to low salt DME containing 2% fetal calf serum and incubated for 2-3 days. The media was collected and adjusted to 1 mM $CaCl_2$ and 5 mM benzamidine. Soluble core-2 β1,6-GlcNAcT containing an HPC4 epitope tag was purified from the conditioned medium (60 ml) using a HPC4-mAb affinity

column (3.5 ml column of 5 mg/ml HPC4-mAb coupled to Ultralink® Biosupport Medium) at 4°C as described in Mehta et al., Blood, 90:2381-2389, 1997. The purified enzyme was stabilized by adding 0.1% BSA and the enzyme was concentrated using Centricon-30 ultrafiltration tubes (Amicon, Beverly, MA). The purified enzyme was used directly or aliquoted and stored at -20°C. The activity (8.2 μmol/h/ml) was stable at -20°C for at least 2 months. Core-2 β1,6-GlcNAcT assays were performed using 1 mM Galβ1→3GalNAcα-pNp (Toronto Research Chemicals Inc., Canada) and 1 mM UDP-[^{3}H]GlcNAc (specific activity 1000 cpm/nmol). The assays were carried out at 37°C with 2.5-10 μl of the purified enzyme for 30 min or 25 μl of cell culture media for 2-3 h in a total volume of 50 μl of 50 mM Na-cacodylate, pH 7.0. The radiolabeled reaction product was separated from the radiolabeled donor using Sep Pak cartridges (Waters, Milford, MA).

Neutrophil Isolation and Labeling

Human neutrophils were isolated from healthy volunteers as described in Zimmerman et al., J. Clin Invest., 76:2235-2246, 1985, and labeled with Calcein-AM (Molecular Probes, Inc., Eugene, OR) according to the manufacturer's instructions.

Neutrophil Adhesion Assay

The adhesion assay was performed essentially as described in Ushiyama et al., J. Biol. Chem., 268-15229-15237, 1993, with the following modifications. Calcein-labeled neutrophils were used.

sPS was coated directly on wells of Immulon 1 microtiter plates by incubating the wells with 2 μg/ml of sPS in 0.1 M sodium carbonate buffer at 4°C overnight (100 μl/well). For GSP-6 inhibition, the wells were preincubated with 50 μl of different dilutions of GSP-6 in HBSS containing 0.1% HSA at room temperature for 15 min. In control experiments, wells were preincubated with mAbs against P-selectin. In other controls, mAbs against PSGL-1 or fluid-phase sPS were preincubated with 25 μl of the cell suspension at room temperature for 15 min. The neutrophils (25 μl) were then added to the sPS-coated wells. The number of adherent cells was quantified using an fmax fluorescence plate reader (Molecular Devices).

Example 2

In a second embodiment, the series of synthesis steps are the same as Example 1 except for the first step. In this embodiment of the invention, represented in Figures 2A and 2B, the presynthesized peptide (which comprises at least one tyrosine and at least one amino acid residue to which a glycan can be attached) is provided. The initially provided peptide of this embodiment lacks tyrosine residues which are sulfated and lacks a GalNAc linked thereto. The peptide which may be produced by a commercial peptide synthesizer is then exposed to a sequential series of sugar donors and corresponding sugar transferases to synthesize an oligosaccharide group linked to the O-linking residue of the peptide. In this example, the step (1) comprises exposing the peptide to UDPGalNAc in the presence of α-GalNAcT to add GalNAc in α-linkage to the

serine or threonine residue (or other O-linking amino acid). If the peptide comprises more than one O-linking residue, in this method the GalNAc may or may not be added to only one of the O-linking residues. The subsequent steps are the same as for Example 1.

Example 3

In an alternative embodiment, represented in Figures 3A and 3B, the peptide is created in the same manner as the peptide in Example 1 except for the sulfation step. In this embodiment, as the peptide is synthesized on a commercial peptide synthesizer, one or more sulfated f-moc or t-boc tyrosine derivatives are introduced into the synthesis cycle at appropriate stages to incorporate the tyrosine residues into the peptide at specific positions. Thus, before the oligosaccharide synthesis steps occur, the peptide has already been sulfated at the one or more tyrosine residues. The synthetic peptide with the one or more sulfated tyrosine residues is released under mild acid conditions to preserve the integrity of the sulfated tyrosine residues. The tetraacetylated GalNAc is unblocked as described above. The introduction of the tetraacetylated GalNAc residue at the specific position in the peptide ensures that no other O-linkage residue in the peptide is modified. The specific introduction of the sulfated tyrosines at one or more designated positions into the peptide assures that the one or more sulfated tyrosines are present in quantitative and stoichiometric levels.

Example 4

In an alternative embodiment, represented in Figure 4, the glycosulfopeptide may be constructed in the same manner as the glycosulfopeptide in Example 3 except the peptide may be initially synthesized using an f-moc or t-boc derivative of tetraacetylated GlcNAc rather than a derivative of tetraacetylated GalNAc. In this embodiment, the O-glycan is constructed by deacetylating the peptide with a weak base in organic solvent such as sodium methoxide followed by steps 4-6 of Example 1 to add Gal, NeuAc and Fuc to the GlcNAc to create the O-glycan linked to the peptide. In this embodiment, the O-glycan is a sialyl Lewis^x group O-linked directly to the O-linkable amino acid residue of the peptide.

Example 5

In an alternative embodiment a sulfate can be added to a specific tyrosine residue. The process shown in Figures 1A and 1B and described in Example 1 is duplicated except for the initial step and final step. In this embodiment, the peptide is initially synthesized using one or more phosphorylated tyrosine residues (Tyr-PO_3^-), for example, at the 46, 48 or 51 positions. The peptide is synthesized with at least one non-phosphorylated tyrosine residue, for example, at least one of the 46, 48, or 51 positions. The peptide therefore has at least one phosphorylated tyrosine residue and at least one non-phosphorylated residue.

This peptide is treated using the same steps described in Example 1, including treating the glycophosphopeptide with a

tyrosylprotein sulfotransferase to add a sulfate group (SO_3^-) to the unoccupied tyrosine residue. The glycosulfophosphopeptide which results is then treated with alkaline phosphatase in a manner well known in the art. This treatment removes the phosphate groups from the tyrosines thereby leaving the sulfate group on the tyrosine to provide the final product, the glycosulfopeptide.

Example 6

To test whether a synthetic glycosulfopeptide of the present invention interacts with P-selectin, a series of affinity columns containing recombinant soluble P-selectin (sPS) at different coupling densities were prepared. The different column densities allow estimation of the relative affinities of synthetic glycosulfopeptides for P-selectin. A radiolabeled glycosulfopeptide having the same structure as glycosulfopeptide-6 (GSP-6) in Figure 1 was applied to immobilized sPS in Ca^{2+} containing buffer (Fig. 5). The elution of the GSP-6 was retarded on columns containing 1.3 and 1.6 mg/ml sPS. The GSP-6 bound to the column containing 2.0 mg/ml sPS and could be eluted with EDTA. Other glycopeptides lacking the sulfates on tyrosines or glycosulfopeptides lacking the sLe^x determinant had no detectable affinity for sPS. The results demonstrate the dual importance of sulfated tyrosines and sLe^x for binding. Glycosulfopeptides lacking the fucosyl residue also did not bind detectably to immobilized sPS. These results demonstrate that both the sialic

acid and the fucose in the sLe^x group are necessary for high affinity binding of GSP-6 to immobilized sPS.

The dissociation constant (K_d) for binding of GSP-6 to soluble sPS was determined using an equilibrium gel filtration technique. Different amounts of fluid-phase sPS (25-1000 pmol) were loaded into small gel filtration columns equilibrated with $^{35}SO_3$-glycosulfopeptide-6 in Ca^{2+}-containing buffer. The binding data were plotted to derive the equilibrium binding constant, yielding an estimated K_d of about 350 nM. Binding of GSP-6 to sPS was inhibited with EDTA and with the inhibitory anti-P-selectin mAb G1, which binds to the lectin domain of P-selectin. Binding was not inhibited with anti-P-selectin mAb s12, which binds to one of the consensus repeats of P-selectin. These results demonstrate that GSP-6 binds with relatively high affinity to sPS in a Ca^{2+}-dependent manner.

The ability of GSP-6 to inhibit neutrophil adhesion to P-selectin was tested in microtiter wells coated with sPS (Figure 5). First validated was the specificity of adhesion. Adhesion was inhibited by EDTA and the anti-P-selectin mAb G1, but not by the anti-P-selectin monoclonal antibody S12. Adhesion was also inhibited by PL1, a mAb directed to an N-terminal epitope of PSGL-1 that blocks binding of PSGL-1 to P-selectin. In contrast, PL2, which recognizes an epitope within the mucin decapeptide repeats of PSGL-1, did not inhibit adhesion. These results demonstrate that adhesion in this assay requires binding of PSGL-1 to sPS. Low concentrations of fluid-phase sPS (5.67 μM) inhibited neutrophil

adhesion. A similar concentration of GSP-6 (4.7 μM) also significantly inhibited neutrophil adhesion to immobilized sPS. In marked contrast, a pure sLe^x-containing tetrasaccharide (NeuAcα2→3Galβ1→4[Fucα1→3]GlcNAc) only minimally inhibited neutrophil adhesion even at very high concentrations (5.3 mM). Taken together, these results demonstrate that GSP-6 binds specifically to P-selectin and strongly inhibits PSGL-1-dependent neutrophil adhesion to P-selectin.

Example 7

Examples of various oligosaccharide R groups which may comprise the O-glycan of the glycosulfopeptides contemplated herein are shown in Figures 6A, 6B and 6C. R_1 shown in Figure 6A may be synthesized using the steps described in Example 1, for example.

R_2 is like R_1 except a NeuAc group has been added in an α2,3 linkage via α2,3-ST in the presence of CMPNeuAc to the Gal linked to the GalNAc.

R_3 is like R_1 except the Gal has been linked to the GlcNAc in a β1,3 linkage via β1,3-GalT and Fuc has been linked to the GlcNAc in an α1,4 linkage via α1,4-FT.

R_4 is like R_3 except a NeuAc group has been added in an α2,3 linkage via α2,3-ST in the presence of CMPNeuAc to the Gal linked to the GalNAc.

R_5, R_6, R_7 and R_8 are like R_1, R_2, R_3, and R_4, respectively, except a sulfate group has been linked to the GlcNAc.

R_9 and R_{11} are like R_1 and R_7, respectively, except they are lacking a Gal in β1,3 linkage to the GalNAc.

R_{10} is like R_9 but has a sulfate group linked to the GlcNAc.

R_{12} is like R_1 but has a sialyl Lewis^x group in β1,3 linkage to the terminal Gal group.

R_{13} is like R_{12} but has a NeuAc in α2,3 linkage to the Gal linked to the GalNAc.

R_{14} is like R_{12} except the terminal NeuAc is replaced with a sialyl Lewis^x group in β1,3 linkage to the terminal Gal group.

R_{15} is like R_{14} but has a NeuAc in α2,3 linkage to the Gal linked to the GalNAc.

Groups R_1 - R_{15} are examples of O-glycans which may comprise the glycosulfopeptide contemplated herein. It will be understood, by a person of ordinary skill in the art that these R groups are only representative of the many O-glycans which may be used to synthesize the glycosulfopeptides of the present invention.

<u>Example 8</u>

As noted above, the present invention in its most basic form contemplates a dipeptide comprising a sulfate group linked to a first amino acid of the dipeptide and a glycan linked to a second amino acid, wherein the glycan is a sialyl Lewis^x group or comprises a sialyl Lewis^x group as a portion thereof. Preferably, the glycan is an O-glycan O-linked to the peptide. The first amino acid, to which the sulfate is attached, is tyrosine (Tyr). The second amino acid, to which the O-glycan is linked, is preferably

a threonine (Thr) or serine (Ser) residue but may be any other amino acid residue to which an O-glycan can be linked in O-linkage (for example, tyrosine, hydroxyproline or hydroxylysine).

The present invention further contemplates that the glycan may be linked in N-linkage to the peptide via an amino acid such as aspartic acid, asparagine, glutamic acid, glutamine, arginine, lysine and cysteine. The present invention contemplates that the peptide may be covalently derivatized to contain the glycan. Examples of such dipeptides defined herein are shown in Figure 7 as formulas A and B wherein X_A represents a threonine, serine, or other residue to which the glycan may be linked, and R represents the R groups R_1-R_{15} defined in Example 7 (and shown in Figures 6A-6C). R, of course, may be another O-glycan not shown in Figures 6A-6C if it enables the peptide to function in accordance with the present invention, i.e., binds with high affinity to P-selectin.

The present invention further contemplates peptides such as those represented as formulas A and B in Figure 8. Glycosulfopeptides in Figure 8 are similar to the glycosulfopeptides represented in Figure 7 except one or more amino acid residues represented by $[X_B]_K$ may be disposed between the sulfate-linked residue (tyrosine) and the O-glycan linked residue X_A (i.e., Ser, Thr or other O-linkable residue, natural or derivatized). X_B represents any amino acid and k represents the number of amino acid residues X_B in a sequence which, in a preferred embodiment, can be from 0-12. Where k=0, the peptides are those shown in Figure 7. Where k=2 or more, the 2 or more

2 or more residues which comprise X_B may be the same amino acid or different amino acids.

A particularly preferred embodiment of the invention is shown below as Compound I, wherein the glycosulfopeptide comprises a heptapeptide having a sulfated tyrosine residue at the N-terminal end and an O-glycosylated linking residue (such as Thr or Ser) at the C-terminal end of the peptide. The GSP comprises five intermediate amino acids represented as X_1, X_2, X_3, X_4, and X_5 as shown below. In one embodiment, X_1 is aspartic acid, X_2 is phenylalanine, X_3 is leucine, X_4 is proline and X_5 is glutamic acid. Preferably, the heptapeptide comprises a component (an amino acid or glycosyl component) which distinguishes it from a fragment of naturally-occurring or recombinantly expressed forms of PSGL-1, i.e., a fragment which could not be obtained from degradation of PSGL-1.

For example, the GSP may comprise fewer than seven amino acids wherein one or more of X_1-X_5 is not present. Alternatively, any one or more of X_1-X_5 may be substituted with a different amino acid, preferably one which has similar functional characteristics as the amino acid being substituted for. Alternatively, X_1-X_5 may comprise repeats of the same amino acid, e.g., five glycine residues. In an especially preferred version the peptide contains one proline residue in the position between tyrosine and the O-linking residue to which the glycan is linked.

(I)

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                          NeuAc
                           |α2,3
                           ↓
                          Gal
                           |β1,4
                     α1,3  ↓
                  Fuc────→ GlcNAc
                           |
               β1,3        ↓β1,6
SO3-          Gal────→ GalNAc
|                          |α1
Tyr-X1-X2-X3-X4-X5-(O-linking aa, e.g., Thr or Ser)
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Example 9

The glycosulfopeptides represented by formulas A and B in Figure 9 are essentially the same as glycosulfopeptides in Figure 8 except each peptide may be extended in an N-terminal and/or C-terminal direction (A and B, Figure 9 respectively) by j or n additional amino acid residues [X_c] and/or [X_D], respectively, where j and n may be, in a preferred version of the invention, from 0-12, and where [X_c] and [X_D] may represent any amino acid. For example, X_c or X_D may each comprise one or more amino acids which are the same, or may comprise different amino acids.

Further, it is contemplated herein that the glycosulfopeptide may comprise more than one sulfated tyrosine residue as shown in Figures 10A and 10B. Figures 10A-B shows a number of preferred glycosulfopeptides A-N having one, two, or three sulfated tyrosine residues. Glycosulfopeptides A and H, for example, comprise three tyrosine residues each having a sulfate group linked thereto. Glycosulfopeptides B, C, D, I, J, and K each have two sulfated

tyrosine residues. Glycosulfopeptides E, F, G, L, M, and N, each have one sulfated tyrosine group. The glycosulfopeptides represented in Figures 10A and 10B are intended to represent only a subset of the compounds contemplated herein as will be appreciated by a person of ordinary skill in the art and may have more or fewer amino acid residues.

Preferably, the glycosulfopeptide comprises an O-glycan comprising a β1,6 linkage to GalNAc. In a particularly preferred embodiment of the present invention, the O-glycan of the glycosulfopeptide is core-2 based.

Example 10

To test whether the specific type of linkage to the GalNAc is important for binding of GSP-6 to immobilized sPS, a novel glycosulfopeptide that is isomeric in structure to GSP-6 was synthesized. This glycosulfopeptide, designated core-1 GSP-6 (Figure 11), has sLe^x on an extended core-1 based O-glycan (C1-O-sLe^x) rather than on a core-2 based (β1,6 linked) O-glycan. A key step in the synthesis of core-1-GSP-6 is the addition of GlcNAc in β1-3 linkage to the Gal residue in the core-1 O-glycan by a recombinant β1,3-GlcNAcT from *Neisseria meningitidis IgtA* (Blixt et al., Glycobiology, In Press, 1999). This glycopeptide was subsequently modified by the action of β1,4-GalT, α2,3-SialylT and α1,3-FucT to generate a glycopeptide which has sLe^x on the extended core-1 O-glycan. This compound was converted to core-1 GSP-6 by

action of TPST-1. Mass spectral analysis confirmed the predicted size of the final product.

Unexpectedly, core-1 GSP-6 did not bind to immobilized sPS. To confirm the presence of sLe^x on the extended core-1 O-glycan, ELISAs were performed using 2H5, a monoclonal antibody that recognizes the sLe^x determinant (Sawada et al., Biochem. Biophys. res. Commun., 193:337-347, 1993). 2H5 bound to immobilized GP-6 and core-1 GP-6, but not to a control glycopeptide GP-2, which lacks sLe^x (data not shown). This verifies the expression of sLe^x determinants on core-1 GP-6. These results demonstrate that preferably the sLe^x glycan is β1,6 linked for binding the glycosulfopeptide-6 to immobilized sPS.

Utility

The present invention provides a method for the treatment of a patient afflicted with inflammatory diseases wherein such disease states may be treated by the administration of an effective amount of a compound of the present invention to a patient in need thereof. The present invention further provides a method of treating a patient to promote an inflammatory response by treating the patient with an effective amount of a compound of the present invention.

A therapeutically effective amount of a compound of the present invention refers to an amount which is effective in controlling, reducing, or promoting the inflammatory response. The term "controlling" is intended to refer to all processes wherein

there may be a slowing, interrupting, arresting, or stopping of the progression of the disease and does not necessarily indicate a total elimination of all disease symptoms.

The term "therapeutically effective amount" is further meant to define an amount resulting in the improvement of any parameters or clinical symptoms characteristic of the inflammatory response. The actual dose will be different for the various specific molecules, and will vary with the patient's overall condition, the seriousness of the symptoms, and counterindications.

As used herein, the term "subject" or "patient" refers to a warm blooded animal such as a mammal which is afflicted with a particular inflammatory disease state. It is understood that guinea pigs, dogs, cats, rats, mice, horses, cattle, sheep, and humans are examples of animals within the scope of the meaning of the term.

A therapeutically effective amount of the compound used in the treatment described herein can be readily determined by the attending diagnostician, as one skilled in the art, by the use of conventional techniques and by observing results obtained under analogous circumstances. In determining the therapeutically effective dose, a number of factors are considered by the attending diagnostician, including, but not limited to: the species of mammal; its size, age, and general health; the specific disease involved; the degree of or involvement or the severity of the disease; the response of the individual patient; the particular compound administered; the mode of administration; the

bioavailability characteristic of the preparation administered; the dose regimen selected; the use of concomitant medication; and other relevant circumstances.

A therapeutically effective amount of the compositions of the present invention will generally contain sufficient active ingredient to deliver from about 0.1 μg/kg to about 50 mg/kg (weight of active ingredient/body weight of patient). Preferably, the composition will deliver at least 0.5 to 10 mg/kg, and more preferably at least 1 μg/kg to 1 mg/kg.

Practice of the method of the present invention comprises administering to a patient a therapeutically effective amount of the active ingredient(s), in any suitable systemic or local formulation, in an amount effective to deliver the dosages listed above. An effective, particularly preferred dosage of the glycosulfopeptide (for example GSP-6) for substantially inhibiting activated neutrophils is 1 μg/kg to 1 mg/kg. The dosage can be administered on a one-time basis, or (for example) from one to 5 times per day.

Preferred amounts and modes of administration are able to be determined by one skilled in the art. One skilled in the art of preparing formulations can readily select the proper form and mode of administration depending upon the particular characteristics of the compound selected the disease state to be treated, the stage of the disease, and other relevant circumstances using formulation technology known in the art, described for example in Remington's Pharmaceutical Sciences, latest edition, Mack Publishing Co.

Pharmaceutical compositions can be manufactured utilizing techniques known in the art. Typically the therapeutically effective amount of the compound will be admixed with a pharmaceutically acceptable carrier.

The compounds or compositions of the present invention may be administered by a variety of routes, for example, orally or parenterally (i.e. subcutaneously, intravenously, intramuscularly, intraperitoneally, or intratracheally).

For oral administration, the compounds can be formulated into solid or liquid preparations such as capsules, pills, tablets, lozenges, melts, powders, suspensions, or emulsions. Solid unit dosage forms can be capsules of the ordinary gelatin type containing for example, surfactants, lubricants and inert fillers such as lactose, sucrose, and cornstarch or they can be sustained release preparations.

In another embodiment, the compounds of this invention can be tabletted with conventional tablet bases such as lactose, sucrose, and cornstarch in combination with binders, such as acacia, cornstarch, or gelatin, disintegrating agents such as potato starch or alginic acid, and a lubricant such as stearic acid or magnesium stearate. Liquid preparations are prepared by dissolving the active ingredient in an aqueous or non-aqueous pharmaceutically acceptable solvent which may also contain suspending agents, sweetening agents, flavoring agents, and preservative agents as are known in the art.

For parenteral administration the compounds may be dissolved in a physiologically acceptable pharmaceutical carrier and administered as either a solution or a suspension. Illustrative of suitable pharmaceutical carriers are water, saline, dextrose solutions, fructose solutions, ethanol, or oils of animal, vegetative, or synthetic origin. The pharmaceutical carrier may also contain preservatives, and buffers as are known in the art.

The compounds of this invention can also be administered topically. This can be accomplished by simply preparing a solution of the compound to be administered, preferably using a solvent known to promote transdermal absorption such as ethanol or dimethyl sulfoxide (DMSO) with or without other excipients. Preferably topical administration will be accomplished using a patch either of the reservoir and porous membrane type or of a solid matrix variety.

As noted above, the compositions can also include an appropriate carrier. For topical use, any of the conventional excipients may be added to formulate the active ingredients into a lotion, ointment, powder, cream, spray, or aerosol. For surgical implantation, the active ingredients may be combined with any of the well-known biodegradable and bioerodible carriers, such as polylactic acid and collagen formulations. Such materials may be in the form of solid implants, sutures, sponges, wound dressings, and the like. In any event, for local use of the materials, the active ingredients usually be present in the carrier or excipient in a weight ratio of from about 1:1000 to 1:20,000, but are not

limited to ratios within this range. Preparation of compositions for local use are detailed in Remington's Pharmaceutical Sciences, latest edition, (Mack Publishing).

Additional pharmaceutical methods may be employed to control the duration of action. Controlled release preparations may be achieved through the use of polymers to complex or absorb the glycosulfopeptide described herein. The controlled delivery may be achieved by selecting appropriate macromolecules (for example, polyesters, polyamino acids, polyvinyl, pyrrolidone, ethylenevinylacetate, methylcellulose, carboxymethylcellulose, or protamine, sulfate) and the appropriate concentration of macromolecules as well as the methods of incorporation, in order to control release.

Another possible method useful in controlling the duration of action by controlled release preparations is incorporation of the glycosulfopeptide molecule or its functional derivatives into particles of a polymeric material such as polyesters, polyamino acids, hydrogels, poly(lactic acid), or ethylene vinylacetate copolymers.

Alternatively, instead of incorporating the GSP into polymeric particles, it is possible to entrap these materials in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatine-microcapsules and poly-(methylmethacylate) microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions,

nano-particles, and nanocapsules), or in macroemulsions. Such techniques are disclosed in the latest edition of Remington's Pharmaceutical Sciences.

U.S. Patent No. 4,789,734 describe methods for encapsulating biological materials in liposomes. Essentially, the material is dissolved in an aqueous solution, the appropriate phospholipids and lipids added, along with surfactants if required, and the material dialyzed or sonicated, as necessary. A good review of known methods is by G. Gregoriadis, Chapter 14. "Liposomes", *Drug Carriers in Biology and Medicine*, pp. 287-341 (Academic Press, 1979). Microspheres formed of polymers or proteins are well known to those skilled in the art, and can be tailored for passage through the gastrointestinal tract directly into the blood stream. Alternatively, the agents can be incorporated and the microspheres, or composite of microspheres, implanted for slow release over a period of time, ranging from days to months. See, for example, U.S. Patent Nos. 4,906,474, 4,925,673, and 3,625,214.

When the composition is to be used as an injectable material, it can be formulated into a conventional injectable carrier. Suitable carriers include biocompatible and pharmaceutically acceptable phosphate buffered saline solutions, which are preferably isotonic.

The term "inflammation" is meant to include reactions of both the specific and non-specific defense systems. A specific defense system reaction is a specific immune system reaction response to an antigen. Examples of a specific defense system reaction include

the antibody response to antigens such as rubella virus, and delayed-type hypersensitivity response mediated by T-cells (as seen, for example, in individuals who test "positive" in the Mantaux test).

A non-specific defense system reaction is an inflammatory response mediated by leukocytes incapable of immunological memory. Such cells include granulocytes, macrophages, neutrophils, etc. Examples of a non-specific defense system reaction include the immediate swelling at the site of a bee sting, the reddening and cellular infiltrate induced at the site of a burn and the collection of PMN leukocytes at sites of bacterial infection (e.g., pulmonary infiltrates in bacterial pneumonias, pus formation in abscesses).

Although the invention is particularly suitable for cases of acute inflammation, it also has utility for chronic inflammation. Types of inflammation that can be treated with the present invention include diffuse inflammation, traumatic inflammation, immunosuppression, toxic inflammation, specific inflammation, reactive inflammation, parenchymatous inflammation, obliterative inflammation, interstitial inflammation, croupous inflammation, and focal inflammation.

It will be appreciated that the present invention will be easily adapted to the diagnosis, monitoring, and treatment of inflammatory disease processes such as rheumatoid arthritis, acute and chronic inflammation, post-ischemic (reperfusion) leukocyte-mediated tissue damage, acute leukocyte-mediated lung injury (e.g.,

Adult Respiratory Distress Syndrome), and other tissue-or organ-specific forms of acute inflammation (e.g., glomerulonephritis).

For reconstitution of a lyophilized product in accordance with this invention, one may employ a sterile diluent, which may contain materials generally recognized for approximating physiological conditions and/or as required by governmental regulation. In this respect, the sterile diluent may contain a buffering agent to obtain a physiologically acceptable pH, such as sodium chloride, saline, phosphate-buffered saline, and/or other substances which are physiologically acceptable and/or safe for use. In general, the material for intravenous injection in humans should conform to regulations established by the Food and Drug Administration, which are available to those in the field.

The pharmaceutical composition may also be in the form of an aqueous solution containing many of the same substances as described above for the reconstitution of a lyophilized product.

The compounds can also be administered as a pharmaceutically acceptable acid- or base- addition salt, formed by reaction with inorganic acids such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, and phosphoric acid, and organic acids such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, and fumaric acid, or by reaction with an inorganic base such as sodium hydroxide, ammonium hydroxide, potassium hydroxide, and organic

bases such as mono-, di-, trialkyl and aryl amines and substituted ethanolamines.

As mentioned above, the products of the invention may be incorporated into pharmaceutical preparations which may be used for therapeutic purposes. However, the term "pharmaceutical preparation" is intended in a broader sense herein to include preparations containing a glycosulfopeptide composition in accordance with this invention, used not only for therapeutic purposes but also for reagent or diagnostic purposes as known in the art, or for tissue culture. The pharmaceutical preparation intended for therapeutic use should contain a "pharmaceutically acceptable" or "therapeutically effective amount" of a GSP, i.e., that amount necessary for preventative or curative health measures. If the pharmaceutical preparation is to be employed as a reagent or diagnostic, then it should contain reagent or diagnostic amounts of a GSP.

Other Utilities

The present invention further comprises a method of producing oligosaccharides. In this method a glycopeptide is synthesized as described previously herein. The glycopeptide is subjected to a β-elimination reaction which causes the cleavage of the linkage between the glycan and the amino acid residue on the peptide to which the glycan was attached thereby producing the free oligosaccharide or glycan. In one version, for example, the β-elimination reaction comprises treating the glycopeptide with 50 mM

NaOH and 1 M sodium borohydride at 50°C for 16 hours. These oligosaccharides can be used, for example, as standards in other analyses.

Another utility of the glycosulfopeptides produced herein is to use specific GSPs with ELISA techniques to enable one to distinguish between monoclonal antibodies which react with core-2 sialyl Lewis^x groups versus those which react with core-1 sialyl Lewis^x groups.

Thus identified, the monoclonal antibodies can be used to characterize epitopes on glycoproteins, for example, to define glycoproteins which have core-2 SLe^x groups versus those which have core-1-SLe^x groups.

Another utility of the GSPs synthesized herein is that the GSPs are excellent acceptors for specific glycosyltransferases. This enables one to assay tissues for the presence therein of specific glycosyltransferases or sulfotransferases.

Another utility of the GSPs synthesized herein is to assay tissues for specific glycosidases which cause release of glycosyl or sulfo groups. HPLC is used to determine whether or not specific GSPs were altered in the assay, thereby indicating the presence or absence of particular glycosidases in the tissue sample.

The present invention may further comprise a method of inhibiting the binding of cells to a selectin comprising exposing the selectin to the a glycosulfopeptide compound described herein in an amount sufficient to bind to a cell binding site on the selectin.

All of the assay methods listed herein are well within the ability of one of ordinary skill in the art given the teachings provided herein.

All references cited herein are hereby incorporated herein in their entirety by reference.

The present invention is not to be limited in scope by the specific embodiments described herein, since such embodiments are intended as but single illustrations of one aspect of the invention and any functionally equivalent embodiments are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

Changes may be made in the formulation and the use of the various compositions described herein or in the steps or the sequence of steps of the methods described herein without departing from the scope of the invention as defined in the following claims.

5. The compound of claim 1 wherein $j = 0$, $k = 0$ to 5 and $n = 0$.

6. The compound of claim 1 wherein X_B comprises proline.

7. The compound of claim 1 wherein X_c comprises tyrosine.

8. The compound of claim 1 comprising one of A-N of Figure 10.

9. The compound of claim 1 further comprising at least one additional sialylated, fucosylated O-glycan linked to an amino acid residue.

10. The compound of claim 1 wherein X_A is an O-linking amino acid.

11. The compound of claim 10 wherein the O-linking amino acid residue is serine or threonine.

12. The compound of claim 1 wherein X_A is an N-linking amino acid.

13. The compound of claim 1 wherein R comprises a β1,6 linkage to a GAlNAc.

14. The compound of claim 1 wherein R is core-2 based.

15. A process for making a glycosulfopeptide compound, comprising:

(a) providing a peptide comprising at least one tyrosine residue and at least one N- or O-linking amino acid residue to which a side group can be linked via an N- or O-linkage, respectively;

(b) linking a GalNAc to the N- or O-linking amino acid residue via an N- or O-linkage, respectively;

(c) enzymatically linking a Gal to the GalNAc;

(d) enzymatically linking a GlcNAc to the GalNAc;

(e) enzymatically linking a second Gal to the GlcNAc;

(f) enzymatically linking a sialic acid to the second Gal;

(g) enzymatically linking a Fuc to the GlcNAc; and

(h) enzymatically linking a sulfate to the tyrosine residue.

16. The method of claim 15 wherein the amino acid to which the side group is attached is an O-linking amino acid.

17. The method of claim 16 wherein the O-linking amino acid is a serine or a threonine.

18. The method of claim 15 wherein the amino acid to which the side group is attached is an N-linking amino acid.

19. The method of claim 15 wherein in step (b), the GalNAc is linked enzymatically.

20. The method of claim 15 wherein in the step of linking the GalNAc to the O-linking amino acid residue is carried out by chemically linking an acetylated GalNAc thereto followed by a deacetylation step.

21. The method of claim 15 wherein step (h) is carried out before or after any one of steps (a)-(g).

22. The method of claim 15 wherein the Gal is linked to the GalNAc via a core-1 β1,3-GalT.

23. The method of claim 15 wherein the sialic acid is neuraminic acid.

24. The method of claim 15 wherein in step (d) the GlcNAc is linked to the GalNAc via a β1,6 linkage.

25. A process for making a glycosulfopeptide compound, comprising:

(a) providing a peptide comprising at least one sulfated tyrosine residue and at least one N- or O-linking amino acid residue;

(b) linking a GalNAc to the N- or O-linking amino acid residue via an N- or O-linkage, respectively;

(c) enzymatically linking a Gal to the GalNAc;

(d) enzymatically linking a GlcNAc to the GalNAc;

(e) enzymatically linking a Gal to the GlcNAc;

(f) enzymatically linking a sialic acid to the Gal; and

(g) enzymatically linking a Fuc to the GlcNAc.

26. The method of claim 25 wherein the N- or O-linking amino acid of step (a) is an O-linking amino acid.

27. The method of claim 25 wherein the O-linking amino acid is a serine or a threonine.

28. The method of claim 25 wherein the N- or O-linking amino acid of step (a) is an N-linking amino acid.

29. The method of claim 25 wherein in step (b) of linking the GalNAc to the amino acid residue, the GalNAc is linked enzymatically.

30. The method of claim 25 wherein in step (b) the linking of the GalNAc to the amino acid residue is carried out by chemically linking an acetylated GalNAc thereto followed by a deacetylation step.

31. The method of claim 25 wherein the Gal is linked to the GalNAc via a core-β1,3-GalT.

32. The method of claim 25 wherein the sialic acid is neuraminic acid.

33. A process for making a glycosulfopeptide compound, comprising:

(a) providing a peptide comprising at least one tyrosine residue, at least one N- or O-linking amino acid residue having a GlcNAc in N- or O-linkage thereto;

(b) enzymatically linking a Gal to the GlcNAc;

(c) enzymatically linking a sialic acid to the Gal;

(d) enzymatically linking a Fuc to the GlcNAc; and

(e) enzymatically linking a sulfate to the tyrosine residue.

34. The method of claim 33 wherein the GlcNAc is linked to the threonine or serine by chemically linking an acetylated GlcNAc thereto followed by a deacetylation step before the Gal is attached to the GlcNAc.

35. The method of claim 33 wherein step (e) is carried out before or after any one of steps (a)-(d).

36. The method of claim 33 wherein the sialic acid is neuraminic acid.

37. A process for making a glycosulfopeptide compound, comprising:

(a) providing a peptide comprising at least one sulfated tyrosine residue and at least one threonine or serine residue having a GlcNAc O-linked thereto;
(b) enzymatically linking a Gal to the GlcNAc;
(c) enzymatically linking a sialic acid to the Gal; and
(d) enzymatically linking a Fuc to the GlcNAc.

38. The method of claim 37 wherein the GlcNAc is linked to the threonine or serine by chemically linking an acetylated GlcNAc thereto followed by a deacetylation step before the Gal is attached to the GlcNAc.

39. The method of claim 37 wherein the sialic acid is neuraminic acid.

40. A process for making an oligosaccharide, comprising: synthesizing a peptide having an oligosaccharide linked thereto by:

(a) providing a peptide having a GalNAc linked to a N- or O-linking amino acid residue of the peptide;

(b) enzymatically linking a Gal to the GalNAc,

(c) enzymatically linking a GlcNAc to the GalNAc,

(d) enzymatically linking a Gal to the GlcNAc,

(e) enzymatically linking a sialic acid to the Gal,

(f) enzymatically linking a Fuc to the GlcNAc; and

cleaving the oligosaccharide from the peptide.

41. The method of claim 40 wherein the N- or O-linking amino acid of step (a) is an O-linking amino acid.

42. The method of claim 41 wherein the O-linking amino acid is a serine or a threonine.

43. The method of claim 40 wherein the N- or O-linking amino acid of step (a) is an N-linking amino acid.

44. The method of claim 40 wherein the Gal in step (b) is linked to the GalNAc via a core-β1,3-GalT.

45. The method of claim 40 wherein the sialic acid is neuraminic acid.

46. A process for making a glycosulfopeptide compound, comprising:

(a) providing a peptide comprising at least one tyrosine residue and at least one N- or O-linking amino acid residue to which a side group can be linked via an N- or O-linkage, respectively;

(b) linking a GalNAc to the N- or O-linking amino acid residue via an N- or O-linkage, respectively;

(c) linking a Gal to the GalNAc;

(d) linking a GlcNAc to the GalNAc;

(e) linking a second Gal to the GlcNAc;

(f) linking a sialic acid to the second Gal;

(g) linking a Fuc to the GlcNAc; and

(h) linking a sulfate to the tyrosine residue.

47. A process for making a glycosulfopeptide compound, comprising:

(a) providing a peptide comprising at least one sulfated tyrosine residue and at least one N- or O-linking amino acid residue;

(b) linking a GalNAc to the N- or O-linking amino acid residue via an N- or O-linkage, respectively;

(c) linking a Gal to the GalNAc;

(d) linking a GlcNAc to the GalNAc;

(e) linking a Gal to the GlcNAc;

(f) linking a sialic acid to the Gal; and

(g) linking a Fuc to the GlcNAc.

48. A process for making a glycosulfopeptide compound, comprising:

(a) providing a peptide comprising at least one tyrosine residue, at least one N- or O-linking amino acid residue having a GlcNAc in N- or O-linkage thereto;

(b) linking a Gal to the GlcNAc;

(c) linking a sialic acid to the Gal;

(d) linking a Fuc to the GlcNAc; and

(e) linking a sulfate to the tyrosine residue.

49. A process for making a glycosulfopeptide compound, comprising:

(a) providing a peptide comprising at least one sulfated tyrosine residue and at least one threonine or serine residue having a GlcNAc O-linked thereto;

(b) linking a Gal to the GlcNAc;

(c) linking a sialic acid to the Gal; and

(d) linking a Fuc to the GlcNAc.

50. A process for making an oligosaccharide, comprising:

synthesizing a peptide having an oligosaccharide linked thereto by:

(a) providing a peptide having a GalNAc linked to a N- or O-linking amino acid residue of the peptide;

(b) linking a Gal to the GalNAc,

(c) linking a GlcNAc to the GalNAc,

(d) linking a Gal to the GlcNAc,

(e) linking a sialic acid to the Gal,

(f) linking a Fuc to the GlcNAc; and

cleaving the oligosaccharide from the peptide.

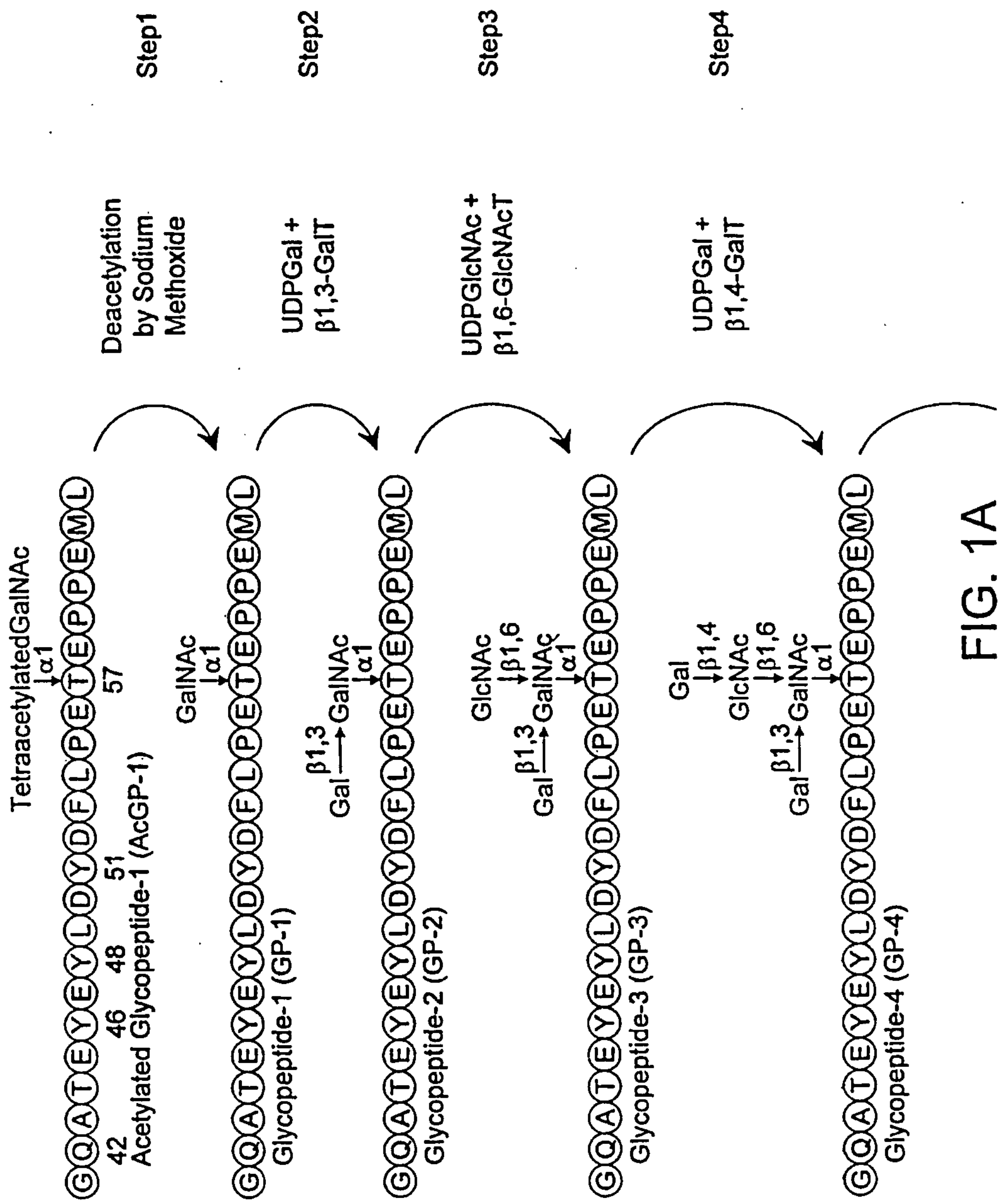
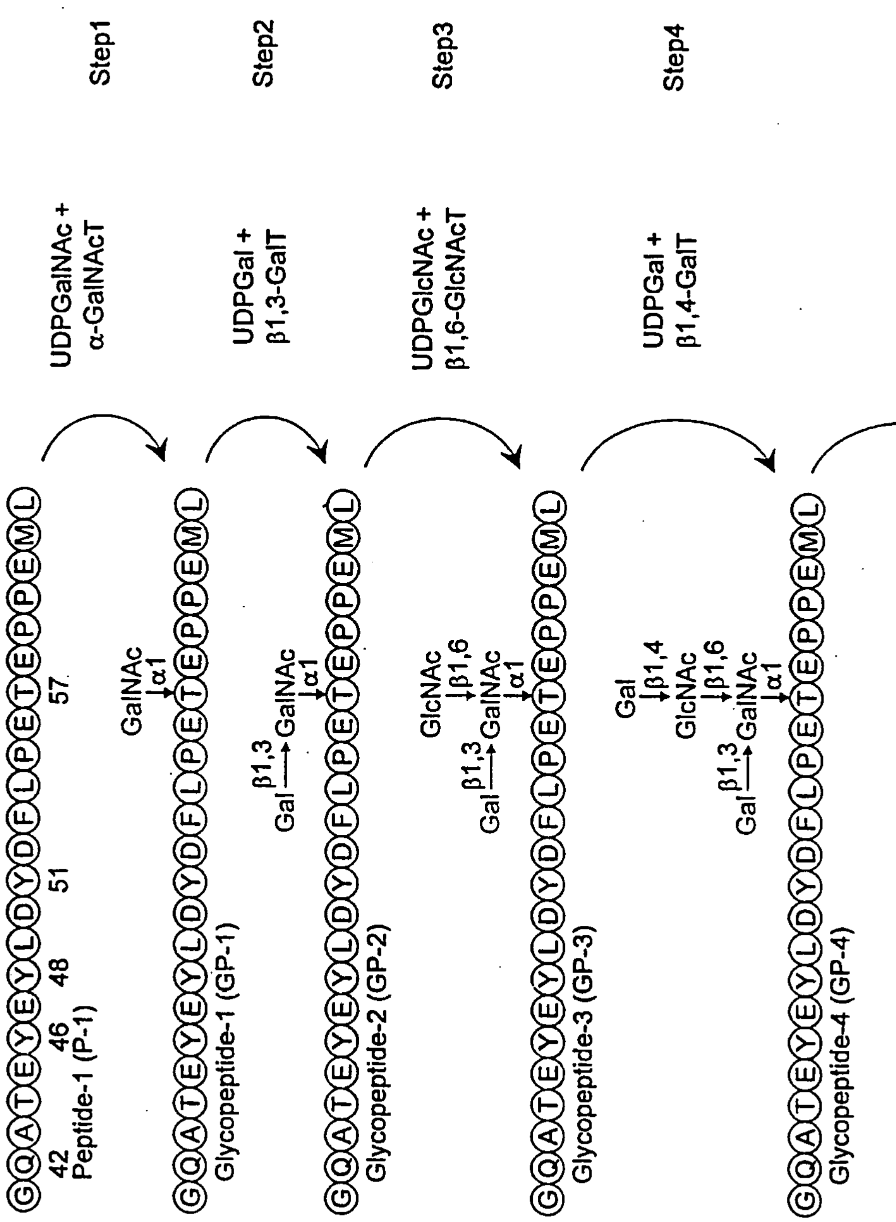
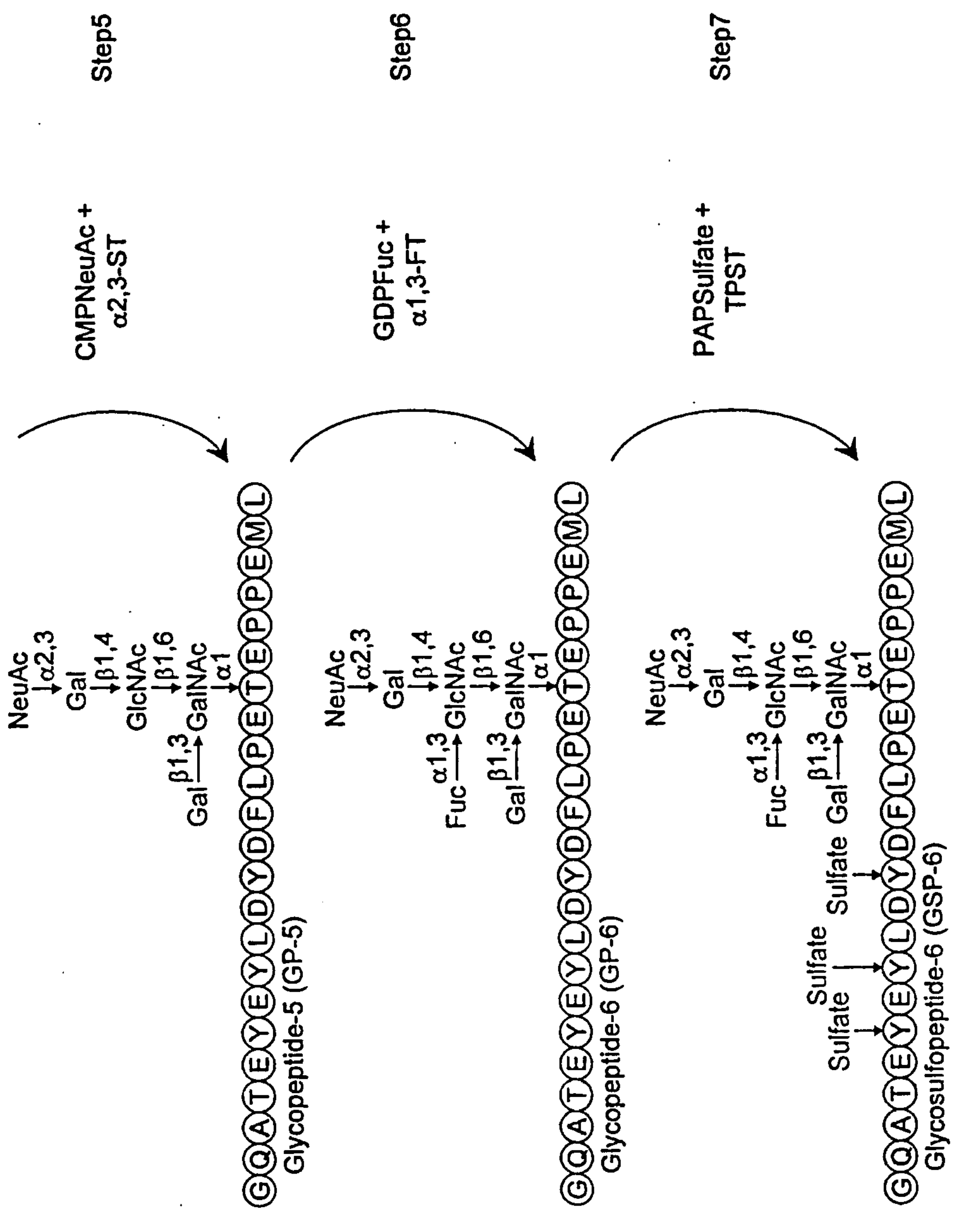

TetraacetylatedGalNAc
α1
42 46 48 51 57
GQATEYEYLDYDFLPETEPPEML
Acetylated Glycopeptide-1 (AcGP-1)
Deacetylation by Sodium Methoxide
Step1
GalNAc
α1
Glycopeptide-1 (GP-1)
UDPGal + β1,3-GalT
Step2
Gal β1,3 GalNAc
α1
Glycopeptide-2 (GP-2)
UDPGlcNAc + β1,6-GlcNAcT
Step3
GlcNAc
β1,6
Gal β1,3 GalNAc
α1
Glycopeptide-3 (GP-3)
UDPGal + β1,4-GalT
Step4
Gal
β1,4
GlcNAc
β1,6
Gal β1,3 GalNAc
α1
Glycopeptide-4 (GP-4)

FIG. 1A



Step1
Step2
Step3
Step4
UDPGalNAc +
α-GalNAcT
UDPGal +
β1,3-GalT
UDPGlcNAc +
β1,6-GlcNAcT
UDPGal +
β1,4-GalT
GQATEYEYLDYDFLPETEPPEML
42 46 48 51 57
Peptide-1 (P-1)
GalNAc
α1
Glycopeptide-1 (GP-1)
Gal β1,3 GalNAc
Glycopeptide-2 (GP-2)
GlcNAc
β1,6
Glycopeptide-3 (GP-3)
Gal
β1,4
Glycopeptide-4 (GP-4)

FIG. 2A



Step5
CMPNeuAc + α2,3-ST
Step6
GDPFuc + α1,3-FT
Step7
PAPSulfate + TPST
NeuAc
α2,3
Gal
β1,4
GlcNAc
β1,6
GalNAc
α1
β1,3
Gal
α1,3
Fuc
Sulfate
GQATEYEYLDYDFLPETEPPEML
Glycopeptide-5 (GP-5)
Glycopeptide-6 (GP-6)
Glycosulfopeptide-6 (GSP-6)

FIG. 2B

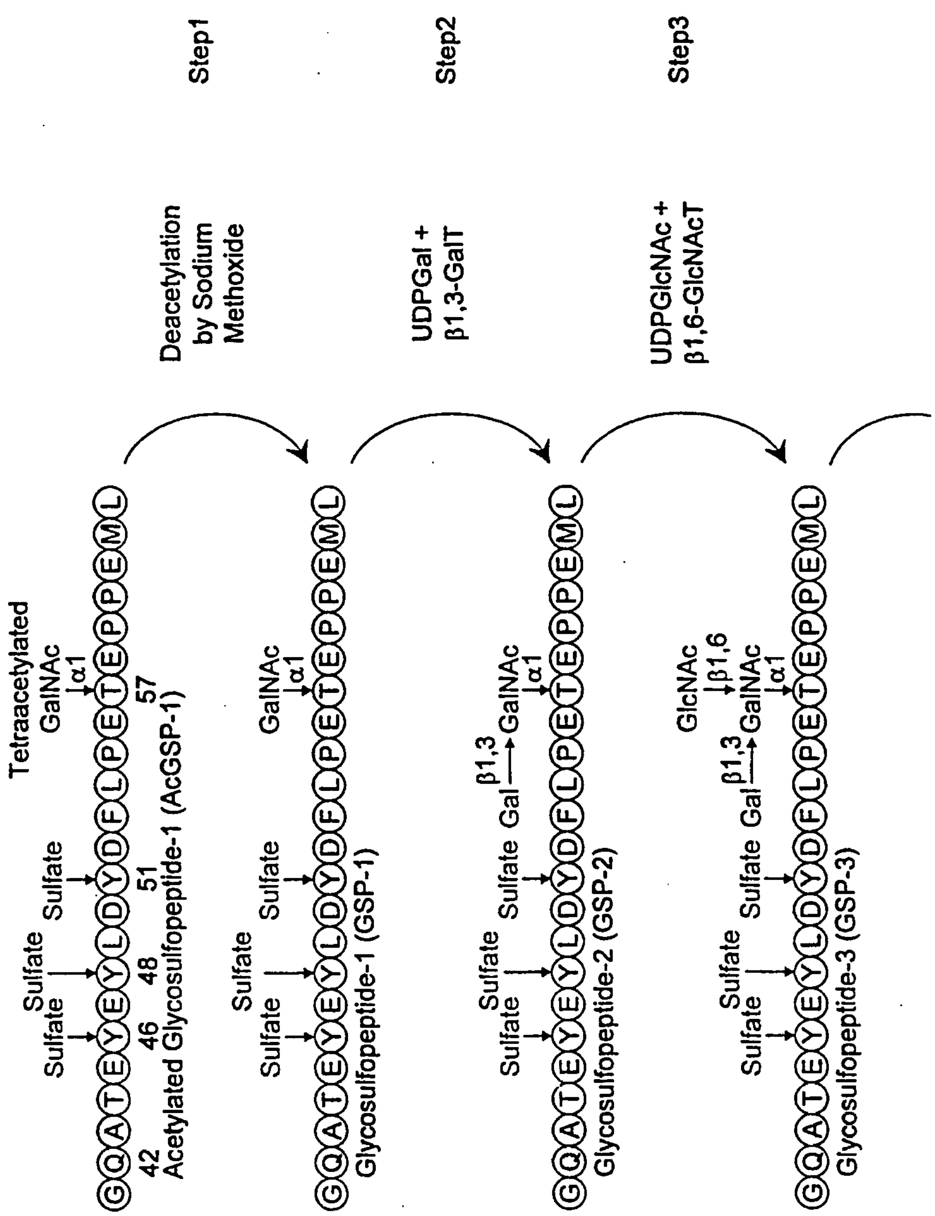

Step1
Step2
Step3
Deacetylation by Sodium Methoxide
UDPGal + β1,3-GalT
UDPGlcNAc + β1,6-GlcNAcT
Sulfate
Sulfate
Sulfate
Tetraacetylated GalNAc
α1
GQATEYEYLDYDFLPETEPPEML
42 46 48 51 57
Acetylated Glycosulfopeptide-1 (AcGSP-1)
GalNAc
Glycosulfopeptide-1 (GSP-1)
Gal β1,3 GalNAc
Glycosulfopeptide-2 (GSP-2)
GlcNAc
β1,6
Glycosulfopeptide-3 (GSP-3)

FIG. 3A

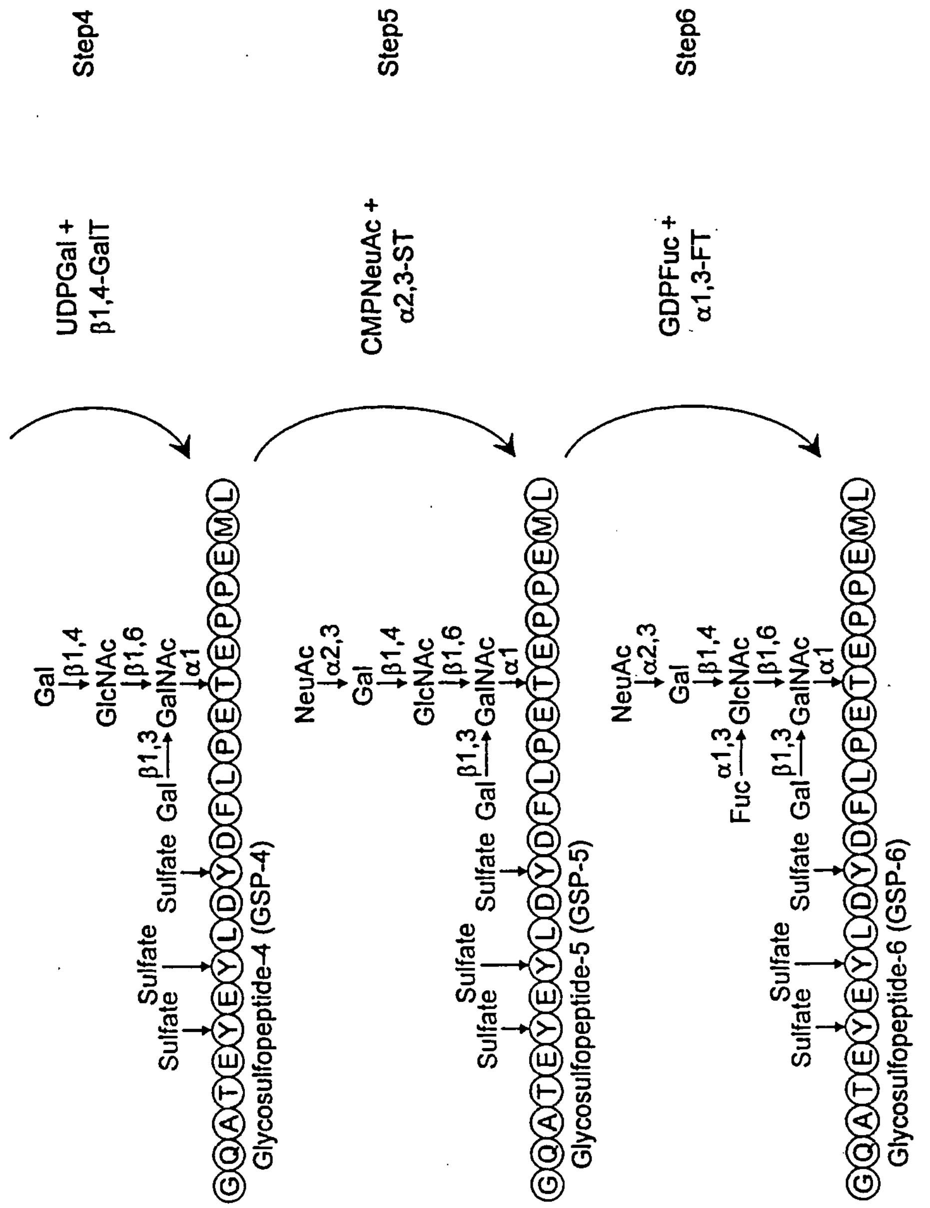

Step4
UDPGal + β1,4-GalT
Step5
CMPNeuAc + α2,3-ST
Step6
GDPFuc + α1,3-FT
Glycosulfopeptide-4 (GSP-4)
Glycosulfopeptide-5 (GSP-5)
Glycosulfopeptide-6 (GSP-6)
Sulfate
Gal
β1,4
GlcNAc
β1,6
GalNAc
α1
β1,3
NeuAc
α2,3
Fuc
α1,3
GQATEYEYLDYDFLPETEPPEML

FIG. 3B

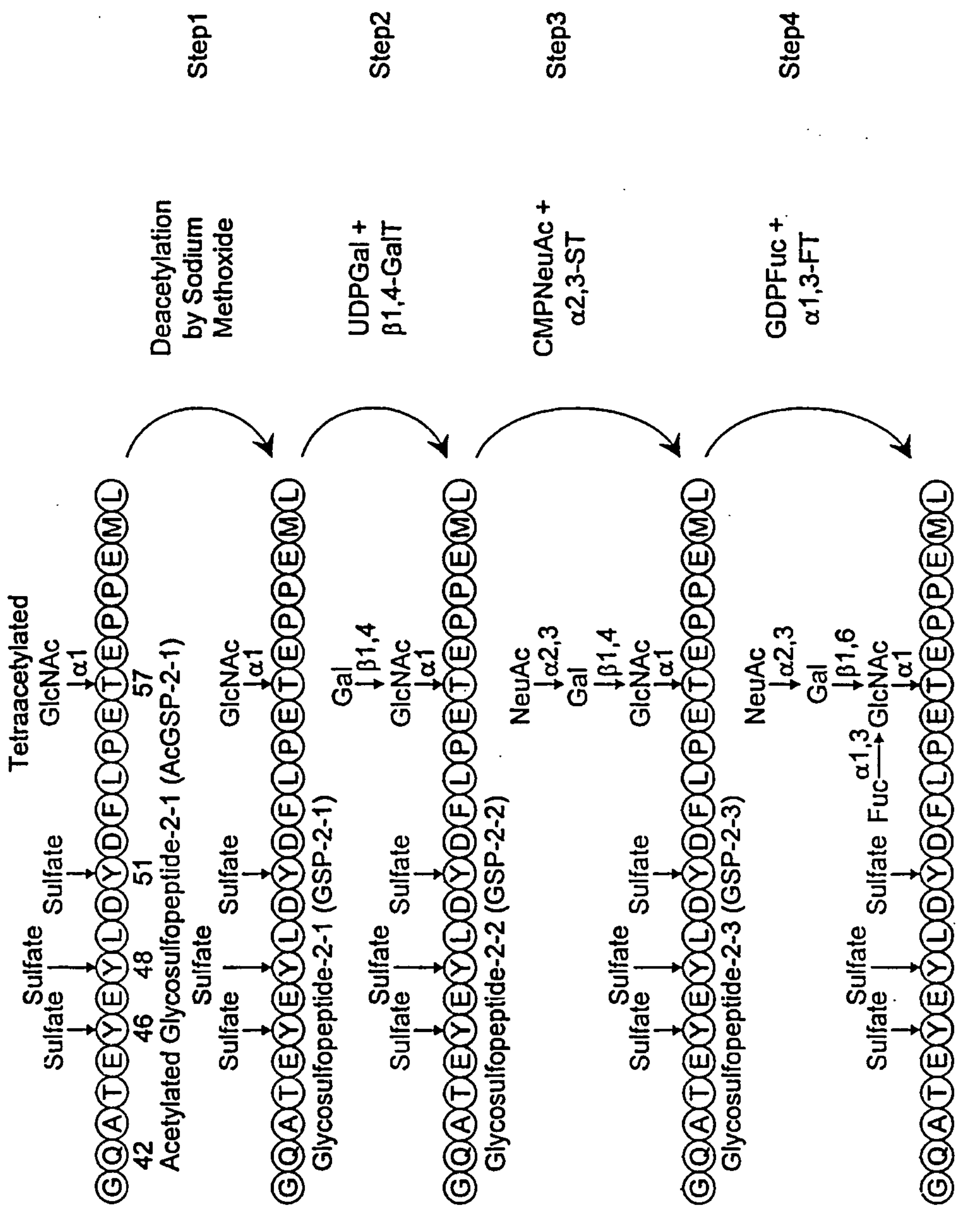
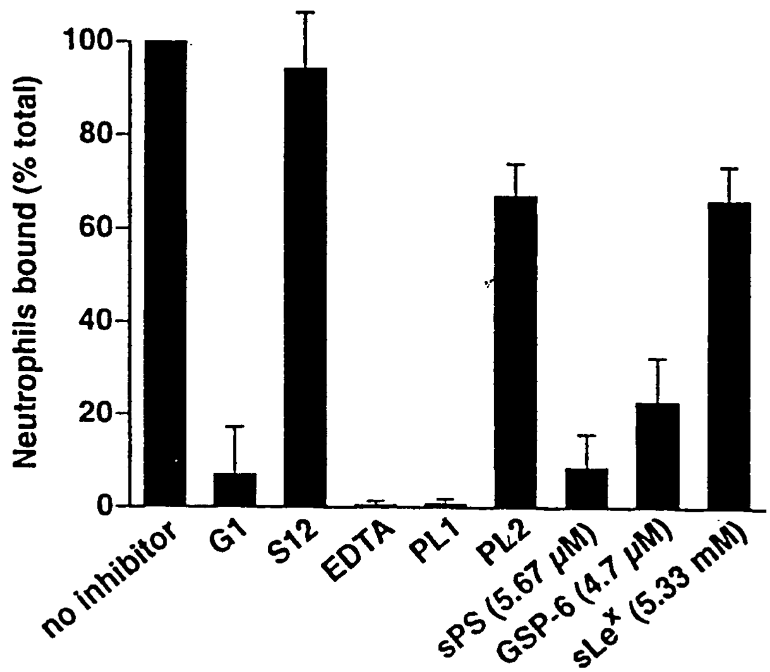

Tetraacetylated GlcNAc
Sulfate
Sulfate
Sulfate
α1
42 46 48 51 57
GQATEYEYLDYDFLPETEPPEML
Acetylated Glycosulfopeptide-2-1 (AcGSP-2-1)
Deacetylation by Sodium Methoxide
Step1
Glycosulfopeptide-2-1 (GSP-2-1)
UDPGal + β1,4-GalT
Step2
Gal
β1,4
GlcNAc
Glycosulfopeptide-2-2 (GSP-2-2)
CMPNeuAc + α2,3-ST
Step3
NeuAc
α2,3
Glycosulfopeptide-2-3 (GSP-2-3)
GDPFuc + α1,3-FT
Step4
β1,6
Fuc
α1,3
Glycosulfopeptide-2-4 (GSP-2-4)

FIG. 4



Effects on Neutrophils Adhesion to Immobilized Soluble P-Selectin

Figure 5

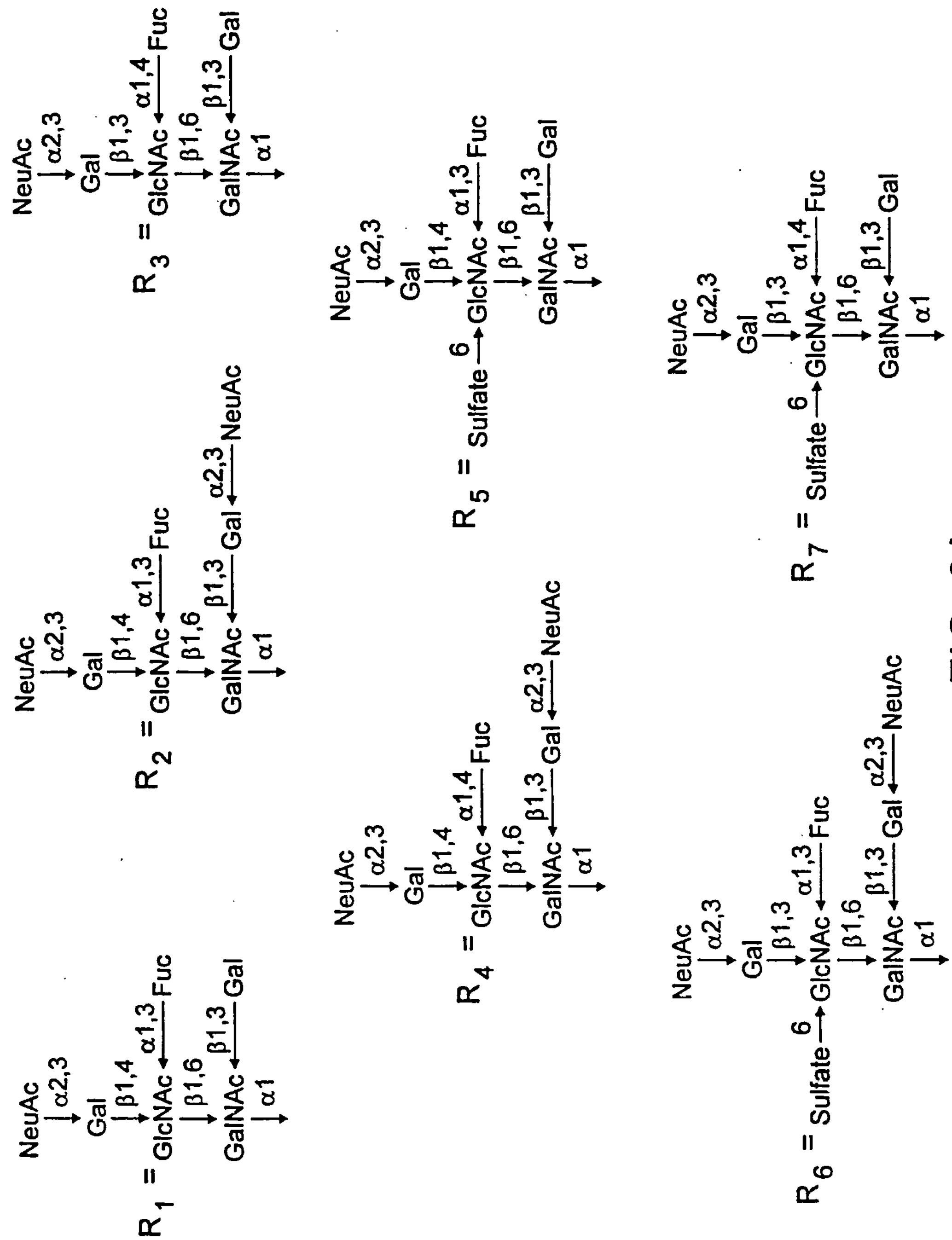


FIG. 6A

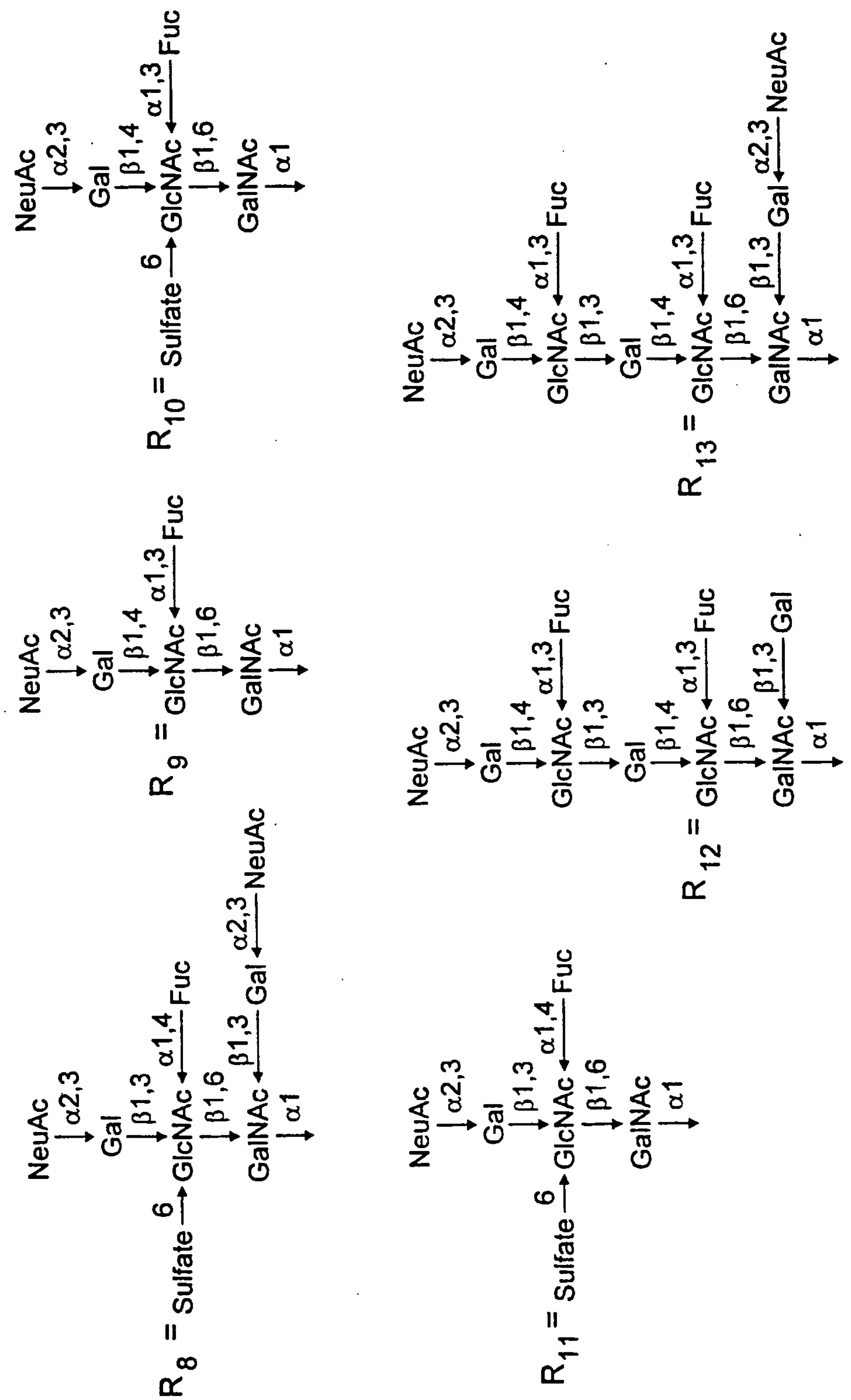

R8 = Sulfate 6 NeuAc α2,3 Gal β1,3 GlcNAc α1,4 Fuc β1,6 β1,3 Gal α2,3 NeuAc GalNAc α1
R9 = NeuAc α2,3 Gal β1,4 GlcNAc α1,3 Fuc β1,6 GalNAc α1
R10 = Sulfate 6 NeuAc α2,3 Gal β1,4 GlcNAc α1,3 Fuc β1,6 GalNAc α1
R11 = Sulfate 6 NeuAc α2,3 Gal β1,3 GlcNAc α1,4 Fuc β1,6 GalNAc α1
R12 = NeuAc α2,3 Gal β1,4 GlcNAc α1,3 Fuc β1,3 Gal β1,4 GlcNAc α1,3 Fuc β1,6 GalNAc β1,3 Gal α1
R13 = NeuAc α2,3 Gal β1,4 GlcNAc α1,3 Fuc β1,3 Gal β1,4 GlcNAc α1,3 Fuc β1,6 GalNAc β1,3 Gal α2,3 NeuAc α1

FIG. 6B

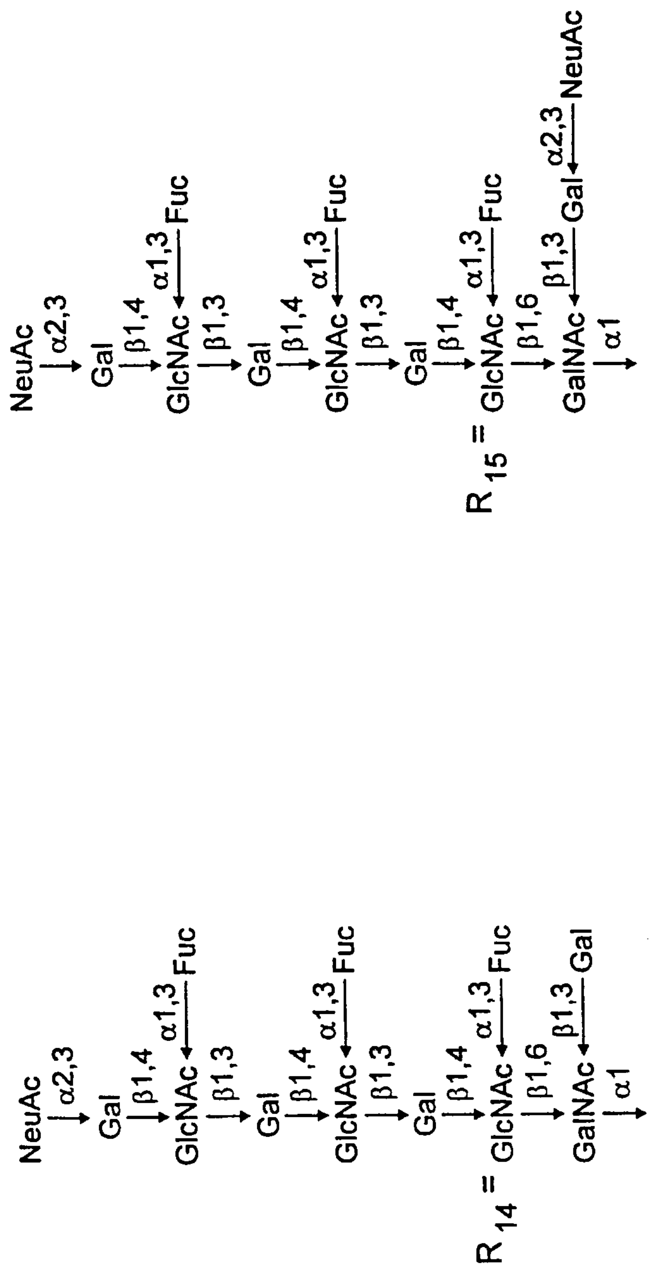

R_{14} =
NeuAc
α2,3
Gal
β1,4
GlcNAc
α1,3
Fuc
β1,3
Gal
β1,4
GlcNAc
α1,3
Fuc
β1,3
Gal
β1,4
GlcNAc
α1,3
Fuc
β1,6
GalNAc
β1,3
Gal
α1
R_{15} =
NeuAc
α2,3
Gal
β1,4
GlcNAc
α1,3
Fuc
β1,3
Gal
β1,4
GlcNAc
α1,3
Fuc
β1,3
Gal
β1,4
GlcNAc
α1,3
Fuc
β1,6
GalNAc
β1,3
Gal
α2,3
NeuAc
α1

FIG. 6C

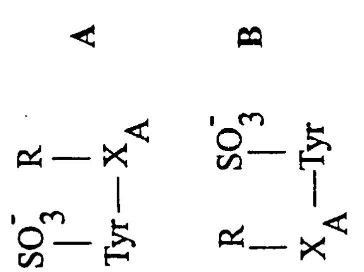


FIG. 7

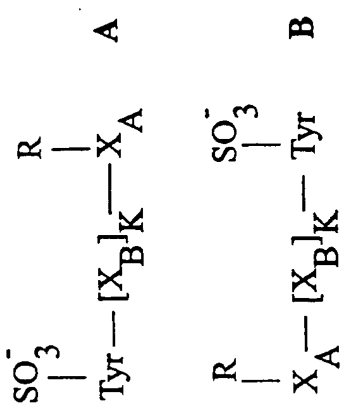


FIG. 8

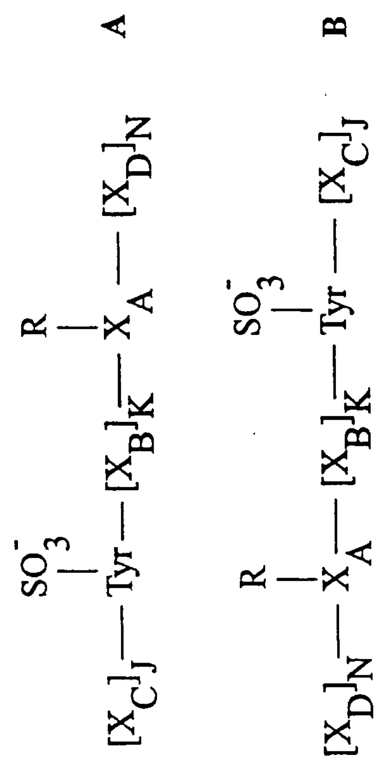
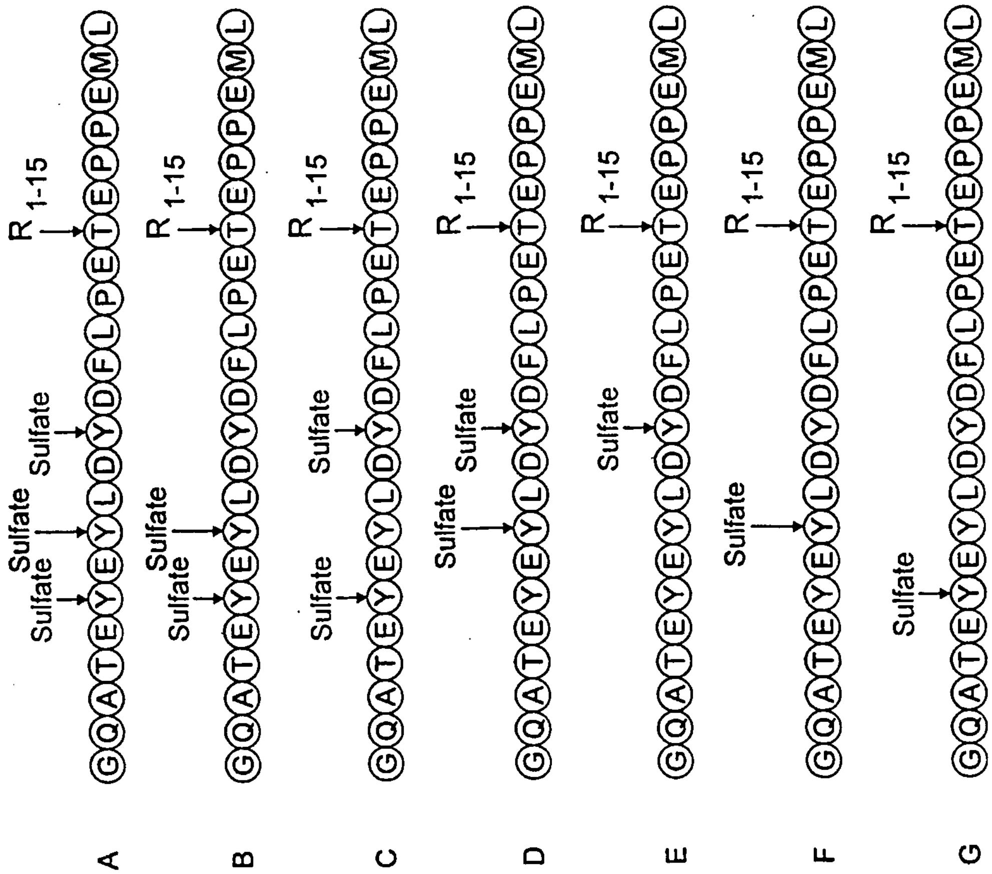


FIG. 9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B GQATEYEYLDYDFLPETEPPEML
C GQATEYEYLDYDFLPETEPPEML
D GQATEYEYLDYDFLPETEPPEML
E GQATEYEYLDYDFLPETEPPEML
F GQATEYEYLDYDFLPETEPPEML
G GQATEYEYLDYDFLPETEPPEML
Sulfate
R 1-15

FIG. 10A

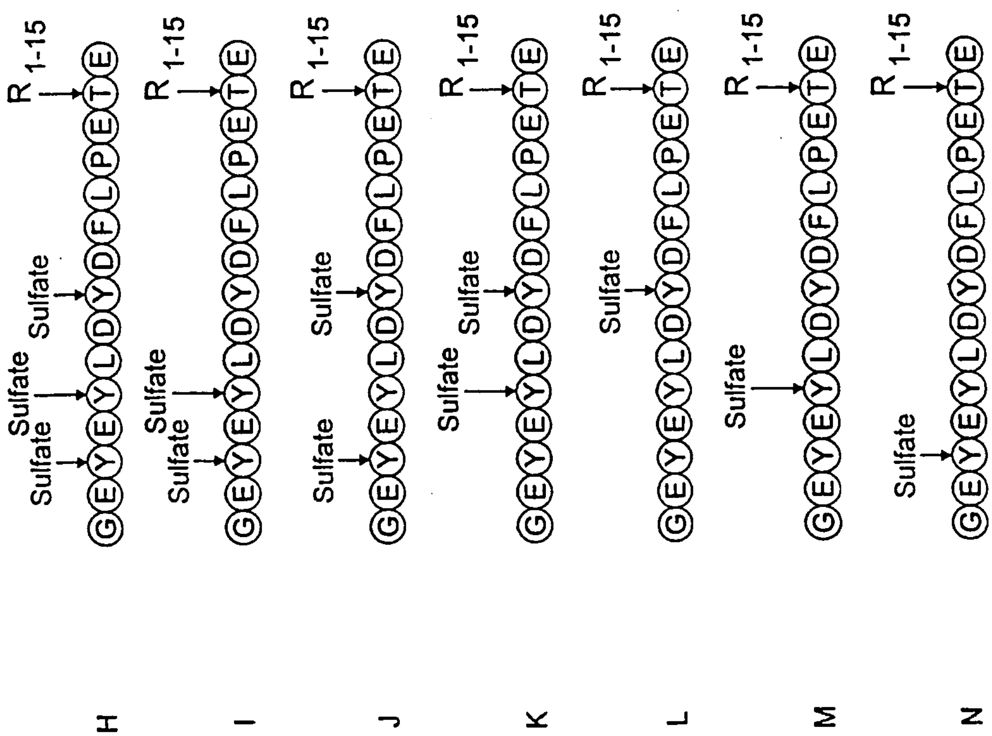

FIG. 10B

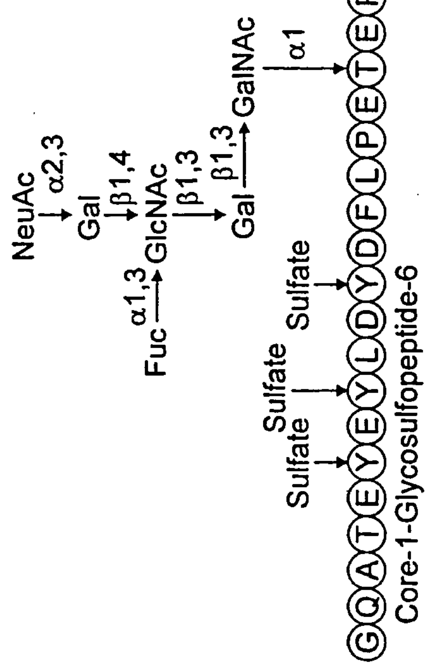
NeuAc
α2,3
Gal
β1,4
Fuc
α1,3
GlcNAc
β1,3
Gal
β1,3
GalNAc
α1
Sulfate
Sulfate
Sulfate
G Q A T E Y E Y L D Y D F L P E T E P P E M L
Core-1-Glycosulfopeptide-6

FIG. 11

SEQUENCE LISTING

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   Met Ala Ser Lys Ser Trp Leu Asn Phe Leu Thr Phe Leu Cys
   1               5                   10

gga tca gca ata gga ttt ctt tta tgt tct cag cta ttt agt att   149
Gly Ser Ala Ile Gly Phe Leu Leu Cys Ser Gln Leu Phe Ser Ile
15                  20                  25

ttg ttg gga gaa aag gtt gac acc cag cct aat gtt ctt cat aat   194
Leu Leu Gly Glu Lys Val Asp Thr Gln Pro Asn Val Leu His Asn
30                  35                  40

gat cct cat gca agg cat tca gat gat aat gga cag aat cat cta   239
Asp Pro His Ala Arg His Ser Asp Asp Asn Gly Gln Asn His Leu
45                  50                  55

gaa gga caa atg aac ttc aat gca gat tct agc caa cat aaa gat   284
Glu Gly Gln Met Asn Phe Asn Ala Asp Ser Ser Gln His Lys Asp
60                  65                  70

gag aac aca gac att gct gaa aac ctc tat cag aaa gtt aga att   329
Glu Asn Thr Asp Ile Ala Glu Asn Leu Tyr Gln Lys Val Arg Ile
75                  80                  85

ctt tgc tgg gtt atg acc ggc cct caa aac cta gag aaa aag gcc   374
Leu Cys Trp Val Met Thr Gly Pro Gln Asn Leu Glu Lys Lys Ala
90                  95                  100

aaa cac gtc aaa gct act tgg gcc cag cgt tgt aac aaa gtg ttg   419
Lys His Val Lys Ala Thr Trp Ala Gln Arg Cys Asn Lys Val Leu
105                 110                 115
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120                 125                 130

aaa acc aaa gaa ggc aga gat caa cta tac tgg aaa aca att aaa    509
Lys Thr Lys Glu Gly Arg Asp Gln Leu Tyr Trp Lys Thr Ile Lys
135                 140                 145

gct ttt cag tat gtt cat gaa cat tat tta gaa gat gct gat tgg    554
Ala Phe Gln Tyr Val His Glu His Tyr Leu Glu Asp Ala Asp Trp
150                 155                 160

ttt ttg aaa gca gat gat gac acg tat gtc ata cta gac aat ttg    599
Phe Leu Lys Ala Asp Asp Asp Thr Tyr Val Ile Leu Asp Asn Leu
165                 170                 175

agg tgg ctt ctt tca aaa tac gac cct gaa gaa ccc att tac ttt    644
Arg Trp Leu Leu Ser Lys Tyr Asp Pro Glu Glu Pro Ile Tyr Phe
180                 185                 190

ggg aga aga ttt aag cct tat gta aag cag ggc tac atg agt gga    689
Gly Arg Arg Phe Lys Pro Tyr Val Lys Gln Gly Tyr Met Ser Gly
195                 200                 205

gga gca gga tat gta cta agc aaa gaa gcc ttg aaa aga ttt gtt    734
Gly Ala Gly Tyr Val Leu Ser Lys Glu Ala Leu Lys Arg Phe Val
210                 215                 220

gat gca ttt aaa aca gac aag tgt aca cat agt tcc tcc att gaa    779
Asp Ala Phe Lys Thr Asp Lys Cys Thr His Ser Ser Ser Ile Glu
225                 230                 235

gac tta gca ctg ggg aga tgc atg gaa att atg aat gta gaa gca    824
Asp Leu Ala Leu Gly Arg Cys Met Glu Ile Met Asn Val Glu Ala
240                 245                 250

gga gat tcc aga gat acc att gga aaa gaa act ttt cat ccc ttt    869
Gly Asp Ser Arg Asp Thr Ile Gly Lys Glu Thr Phe His Pro Phe
255                 260                 265

gtg cca gaa cac cat tta att aaa ggt tat cta cct aga acg ttt    914
Val Pro Glu His His Leu Ile Lys Gly Tyr Leu Pro Arg Thr Phe
270                 275                 280

tgg tac tgg aat tac aac tat tat cct cct gta gag ggt cct ggt    959
Trp Tyr Trp Asn Tyr Asn Tyr Tyr Pro Pro Val Glu Gly Pro Gly
285                 290                 295

tgc tgc tct gat ctt gca gtt tct ttt cac tat gtt gat tct aca   1004
Cys Cys Ser Asp Leu Ala Val Ser Phe His Tyr Val Asp Ser Thr
300                 305                 310
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acc atg tat gag tta gaa tac ctc gtt tat cat ctt cgt cca tat 1049
Thr Met Tyr Glu Leu Glu Tyr Leu Val Tyr His Leu Arg Pro Tyr
315 320 325

ggt tat tta tac aga tat caa cct acc tta cct gaa cgt ata cta 1094
Gly Tyr Leu Tyr Arg Tyr Gln Pro Thr Leu Pro Glu Arg Ile Leu
330 335 340

aag gaa att agt caa gca aac aaa aat gaa gat aca aaa gtg aag 1139
Lys Glu Ile Ser Gln Ala Asn Lys Asn Glu Asp Thr Lys Val Lys
345 350 355

tta gga aat cct tga aagaaaatcatgaatgaacaaaggtaatatgtctagcac 1193
Leu Gly Asn Pro
360

tgcactgaaaaaggacttctgcatttctgacatagaacactggaatcccagtgaggaat 1252

tctaagtgaacattccttatagaaacctttcacatgaatgactataaactgaagctttta 1311

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Met Val Gly Lys Leu Lys Gln Asn Leu Leu Leu Ala Cys Leu Val Ile
 1               5                  10                  15

Ser Ser Val Thr Val Phe Tyr Leu Gly Gln His Ala Met Glu Cys His
            20                  25                  30

His Arg Ile Glu Glu Arg Ser Gln Pro Val Lys Leu Glu Ser Thr Arg
        35                  40                  45

Thr Thr Val Arg Thr Gly Leu Asp Leu Lys Ala Asn Lys Thr Phe Ala
    50                  55                  60

Tyr His Lys Asp Met Pro Leu Ile Phe Ile Gly Gly Val Pro Arg Ser
65                  70                  75                  80

Gly Thr Thr Leu Met Arg Ala Met Leu Asp Ala His Pro Asp Ile Arg
                85                  90                  95

Cys Gly Glu Glu Thr Arg Val Ile Pro Arg Ile Leu Ala Leu Lys Gln
            100                 105                 110

Met Trp Ser Arg Ser Ser Lys Glu Lys Ile Arg Leu Asp Glu Ala Gly
        115                 120                 125

Val Thr Asp Glu Val Leu Asp Ser Ala Met Gln Ala Phe Leu Leu Glu
    130                 135                 140

Ile Ile Val Lys His Gly Glu Pro Ala Pro Tyr Leu Cys Asn Lys Asp
145                 150                 155                 160

Pro Phe Ala Leu Lys Ser Leu Thr Tyr Leu Ser Arg Leu Phe Pro Asn
                165                 170                 175

Ala Lys Phe Leu Leu Met Val Arg Asp Gly Arg Ala Ser Val His Ser
            180                 185                 190

Met Ile Ser Arg Lys Val Thr Ile Ala Gly Phe Asp Leu Asn Ser Tyr
        195                 200                 205

Arg Asp Cys Leu Thr Lys Trp Asn Arg Ala Ile Glu Thr Met Tyr Asn
    210                 215                 220

Gln Cys Met Glu Val Gly Tyr Lys Lys Cys Met Leu Val His Tyr Glu
225                 230                 235                 240
```

```
Gln Leu Val Leu His Pro Glu Arg Trp Met Arg Thr Leu Leu Lys Phe
                245                 250                 255

Leu Gln Ile Pro Trp Asn His Ser Val Leu His His Glu Glu Met Ile
            260                 265                 270

Gly Lys Ala Gly Gly Val Ser Leu Ser Lys Val Glu Arg Ser Thr Asp
        275                 280                 285

Gln Val Ile Lys Pro Val Asn Val Gly Ala Leu Ser Lys Trp Val Gly
    290                 295                 300

Lys Ile Pro Pro Asp Val Leu Gln Asp Met Ala Val Ile Ala Pro Met
305                 310                 315                 320

Leu Ala Lys Leu Gly Tyr Asp Pro Tyr Ala Asn Pro Pro Asn Tyr Gly
                325                 330                 335

Lys Pro Asp Pro Lys Ile Ile Glu Asn Thr Arg Arg Val Tyr Lys Gly
            340                 345                 350

Glu Phe Gln Leu Pro Asp Phe Leu Lys Glu Lys Pro Gln Thr Glu Gln
        355                 360                 365

Val Glu
    370
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<211> 377
<212> PRT
<213> Homo sapiens
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Met Arg Leu Ser Val Arg Arg Val Leu Leu Ala Ala Gly Cys Ala Leu
 1               5                  10                  15

Val Leu Val Leu Ala Val Gln Leu Gly Gln Gln Val Leu Glu Cys Arg
            20                  25                  30

Ala Val Leu Ala Gly Leu Arg Ser Pro Arg Gly Ala Met Arg Pro Glu
        35                  40                  45

Gln Glu Glu Leu Val Met Val Gly Thr Asn His Val Glu Tyr Arg Tyr
    50                  55                  60

Gly Lys Ala Met Pro Leu Ile Phe Val Gly Gly Val Pro Arg Ser Gly
65                  70                  75                  80

Thr Thr Leu Met Arg Ala Met Leu Asp Ala His Pro Glu Val Arg Cys
                85                  90                  95

Gly Glu Glu Thr Arg Ile Ile Pro Arg Val Leu Ala Met Arg Gln Ala
            100                 105                 110

Trp Ser Lys Ser Gly Arg Glu Lys Leu Arg Leu Asp Glu Ala Gly Val
        115                 120                 125

Thr Asp Glu Val Leu Asp Ala Ala Met Gln Ala Phe Ile Leu Glu Val
    130                 135                 140

Ile Ala Lys His Gly Glu Pro Ala Arg Val Leu Cys Asn Lys Asp Pro
145                 150                 155                 160

Phe Thr Leu Lys Ser Ser Val Tyr Leu Ser Arg Leu Phe Pro Asn Ser
                165                 170                 175

Lys Phe Leu Leu Met Val Arg Asp Gly Arg Ala Ser Val His Ser Met
            180                 185                 190

Ile Thr Arg Lys Val Thr Ile Ala Gly Phe Asp Leu Ser Ser Tyr Arg
        195                 200                 205

Asp Cys Leu Thr Lys Trp Asn Lys Ala Ile Glu Val Met Tyr Ala Gln
    210                 215                 220

Cys Met Glu Val Gly Lys Glu Lys Cys Leu Pro Val Tyr Tyr Glu Gln
225                 230                 235                 240
```

Leu Val Leu His Pro Arg Arg Ser Leu Lys Leu Ile Leu Asp Phe Leu
245 250 255

Gly Ile Ala Trp Ser Asp Ala Val Leu His His Glu Asp Leu Ile Gly
260 265 270

Lys Pro Gly Gly Val Ser Leu Ser Lys Ile Glu Arg Ser Thr Asp Gln
275 280 285

Val Ile Lys Pro Val Asn Leu Glu Ala Leu Ser Lys Trp Thr Gly His
290 295 300

Ile Pro Gly Asp Val Val Arg Asp Met Ala Gln Ile Ala Pro Met Leu
305 310 315 320

Ala Gln Leu Gly Tyr Asp Pro Tyr Ala Asn Pro Pro Asn Tyr Gly Asn
325 330 335

Pro Asp Pro Phe Val Ile Asn Asn Thr Gln Arg Val Leu Lys Gly Asp
340 345 350

Tyr Lys Thr Pro Ala Asn Leu Lys Gly Tyr Phe Gln Val Asn Gln Asn
355 360 365

Ser Thr Ser Ser His Leu Gly Ser Ser
370 375

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<210> 5
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